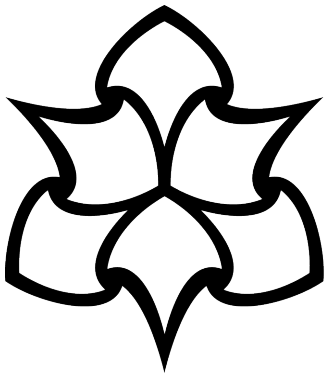

DNA methylation of *TOMM40-APOE* locus and Alzheimer's disease pathology in the prefrontal cortex.

Natalia Ewa Bezuch

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School of Healthcare Science
Manchester Metropolitan University

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Abstract

Introduction: Apolipoprotein E (APOE) encoded by *APOE* gene is an important lipid transport molecule in the central nervous system. Carrying at least one *APOE4* allele is the strongest known genetic risk factor for the sporadic Alzheimer's Disease (AD). APOE isoforms can differentially influence the AD pathogenesis by affecting the synthesis and clearance of the Aβ as well as affecting the neurons independently of the Aβ, for instance by disrupting the mitochondria or cytoskeleton and leading to neurodegeneration and defective repair. *TOMM40* is another gene that have been implicated in AD. Due to its close proximity to the *APOE* gene, it is thought to be in linkage disequilibrium with the *APOE* gene and therefore influence its transcription. *APOE* genotype could also have an effect on the methylation levels, as the 2 SNPs that account for the 3 APOE isoforms, either disrupt or create a CpG site- Rs7412 (C->T) removes a CpG site, whereas rs429358 (T->C) creates an extra CpG site. Previous studies suggested that methylation of the *TOMM40-APOE* locus may influence the APOE protein levels and thus associate with AD pathology. However, it is not fully understood how these genes are transcriptionally regulated.

Aims: The main aim of this study was to investigate *APOE* methylation levels and protein expression in a number of aged (n=32) and AD (n=30) human brains and to test if the gene methylation and protein levels associate with the AD pathology.

Methods: APOE protein levels were estimated using an ELISA. Genotyping was performed using Sequenom MassARRAY iPLEX platform. DNA methylation levels were measured using bisulphite pyrosequencing. Prefrontal cortex samples were stained with rabbit APOE polyclonal antibody and co-stained with mouse Glial Fibrillary Acidic Protein (GFAP) antibody (astrocyte marker) and visualised under fluorescent microscope.

Results: As expected, the number of *APOE*4 carriers was significantly higher in the AD group. *APOE* genotype did not have an effect on the *TOMM40-APOE* locus methylation, nor on the APOE protein expression. APOE protein levels were not significantly associated with AD pathology. TOMM40 CpG2 negatively correlated with APOE protein levels (ρ=-0.270, P=0.042), however the association was no longer significant after adjustment for confounding factors. Methylation of the remaining CpG sites did not correlate with APOE protein expression. *TOMM40-APOE* locus methylation levels did not significantly associate with AD pathology. APOE protein levels positively correlated with longitudinal fluid intelligence, which in turn were negatively associated with Thal score.

Conclusion: *TOMM40-APOE* locus methylation may not be affected by the genotype and the methylation levels do not have a direct effect on the AD pathology. APOE protein levels may have a domain-specific effect in the prefrontal cortex affecting longitudinal fluid intelligence. Further research, preferably in a larger sample group, is necessary to investigate the role of the *TOMM40-APOE* locus methylation in the AD pathology.

1. Introduction

1.1 Dementia and Alzheimer's disease overview (epidemiology and risk factors)

It is estimated that in the UK, 1 in 14 people at the age over 65 suffer from dementia (Prince *et al.,* 2014). As the life expectancy increases, the number of people affected by dementia is estimated to grow in the next decades, from over 850 000 in 2018 to over 2 million in 2050 (Prince *et al.,* 2014). Women have higer life expectancy, therefore also a notably higher disease prevalence in older age group. As the numbers of the affected people increases, it imposes a great burden of the healthcare services and the caregivers. Since the beginning of 2017, dementia has overtaken heart disease as a leading cause of death in the UK. Dementia is currently incurable and the treatments available have limited success in slowing progression of the disease and reducing the symptoms. Alzheimer's disease (AD) is a the most common cause of dementia, accounting for 60-70% of all cases (Burns and Iliffe, 2009). Familial AD is very rare form, inherited in autosomal dominant manner with an early age of onset. Studies on familial AD (FAD), revealed that mutations in APP and components responsible for γ- secretase activity, such as presenilin 1 (PSEN1) and PSEN2 are crucial in the Aβ aggregation (Selkoe and Kopan, 2003). Sporadic AD, on the other hand, is very common, making up over 95% of all AD cases (Masters *et al*., 2015). The onset of sporadic AD is typically late and its cause is unknown, probably due to the heterogeneity of the disease. The main causes of sporadic AD are ageing, together with an intricate interaction between genetic and environmental risk factors. Some of the proposed environmental risk factors include lower reserve capacity of the brain, such as reduced brain size, low educational and occupational attainment, low mental ability in early life and reduced mental and physical activity during late life (Mayeux, 2003; Mortimer *et al*., 2003). The brain reserve capacity is defined by the number of neurons and the dendritic and synaptic connections combined with learning strategies associated with particular lifestyle (Mayeux, 2003). Certain studies have also shown that head injury can be a potential risk factor, however it is not clear

whether it directly influences the pathogenesis resulting in plaque and tangle formation or plainly reduces the brain reserve (Jellinger, 2004). Risk factors associated with vascular disease, such as hypercholesterolemia, hypertension, atherosclerosis, smoking, obesity and diabetes may also play a role; although, it is unclear whether they directly influence the pathogenesis or, by inducing cerebrovascular pathology, they accelerate the onset of clinically silent disease (Mayeux, 2003). Some studies suggest that vitamin B and folate, antioxidants (e.g. vitamin C and E), unsaturated fatty acids and even moderate alcohol intake may have protective effect, however there is not enough evidence for these to be recommended as a method of prevention (Luchsinger and Mayeux, 2004). Even though environmental factors can influence the sporadic AD development, genetics appears to be the most important risk factor, with over 80% of heritability as shown in a large twin-study (Gatz *et al.*, 2006). The most significant genetic risk factor for late onset AD (LOAD) is *APOE*4 allele. As APOE protein is an important cholesterol transport molecule in the cerebrospinal fluid (CSF), it has been suggested that APOE may influence the pathogenesis by affecting the cholesterol transport to the brain. Some studies indicate that high plasma/serum cholesterol levels increase risk of developing AD (Pappolla *et al.*, 2003; Popp *et al.*, 2013). Others suggest that it is an increase in the low- density lipoprotein cholesterol (LDL-C) specifically that contributes to the diseases risk (Kuo *et al.*, 1998). Interestingly, recent findings show that that APOE interacts with LDL receptor (LDLR) in an isoform specific manner, affecting regional brain APOE protein and cholesterol levels and thus influencing cognitive function (Johnson *et al.*, 2014). Although *APOE*4 allele accounts for most of the genetic risk of sporadic AD, other genes may also play a role; however, no single one has been verified with certainty (Blomqvist *et al*., 2006). Recently, numerous genome-wide association studies have shown an association of different SNPs within TOMM40 gene and LOAD (Grupe *et al*., 2007; Feulner *et al*., 2010; Carrasquillo *et al.*, 2009; Harold et al., 2009). As sporadic AD is a complex heterogeneous disease, the susceptibility genes may act in combination together with environmental risk factors and contribution of each of them in the diseases risk may be minor.

1.2 Alzheimer's disease clinical features and pathogenesis

AD is a progressive neurodegenerative disease that usually starts slowly, impairing the episodic memory at first and affecting language, orientation, behaviour, mood and motivation as the disease progresses. The neurodegeneration in AD may start up to 20-30 years before the diseases onset (Davies *et al.,* 1988) and is caused by deposition and toxic activity of extracellular amyloid Beta (Aβ) and abnormally phosphorylated tau protein inside of the neurons. At certain point the threshold is exceeded and the first symptoms start occurring. The first clinical phase of AD is often referred as mild cognitive impairment (MCI), which is characterised by memory loss. MCI can be either a sign of early AD, or could be a benign symptom associated with normal ageing.

The specific mechanisms of the disease pathology are not fully understood. As our knowledge of AD increases, so does the awareness of the complex nature of its pathogenesis.

Amyloid plaque counts positively correlate with the disease's severity, which indicates their importance in the AD pathology. Aβ is obtained from amyloid precursor protein (APP) in a multi-step proteolytic process involving β- and γ-secretases. As Aβ is produced during normal cell metabolism, it has been hypothesised that it is the imbalance between the Aβ synthesis and clearance, that initiates the pathological process of neurodegeneration eventually leading to dementia. Mutations in the key enzymes (PSEN1 and PSEN2) as well as the substrate (APP), necessary for Aβ synthesis, present in the FAD, provide support for this hypothesis. Moreover, people with Down syndrome have an extra copy of APP gene and they develop amyloid plaques earlier in life, which reinforces the hypothesis that APP overexpression initiates Aβ deposition and aggregation. Although, at first, only the plaque-forming large insoluble fibrils were thought to be neurotoxic, some research indicates that soluble oligomers may contribute to inhibited hippocampal long-term potentiation and disrupted synaptic plasticity (Walsh and Selkoe, 2004).

Neurofibrillary tangles are composed of abnormally phosphorylated tau protein. Normal tau is important for microtubule assembly and stability. However, when the

balance between kinases, such as GSK-3β and CDK5 and phosphatases, such as PP-1 and PP-2A which are responsible for tau phosphorylation becomes disrupted, it can lead to intracellular tau hyperphosphorylation and normal tau sequestration (Iqbal *et al*., 2005). As a result of tau sequestration, microtubules become unstable and may disassemble, leading to impaired axonal transport and subsequently compromised neuronal and synaptic function (Iqbal *et al*., 2005). Insoluble tau fibrils aggregating in the tangles further compromise neuronal function. It is not clear whether hyperphosphorylation of tau and formation of tangles is a cause or a result of AD.

There are some studies demonstrating comorbidity of cerebrovascular disease and AD pathology (Cruchaga *et al*., 2013; Mayeux, 2003; Farkas and Luiten, 2001), which suggest they may be an overlap between the cerebrovascular pathology and amyloid plaque formation. Dysfunctional blood vessels may aggravate cognitive impairment by reducing transport of nutrients to neurons and Aβ clearance (Iadecola, 2004). Cerebrovascular pathology associated with ischaemia, leads to APP overexpression and subsequent Aβ deposition (Jendroska *et al*., 1995; Riekse *et al*., 2004; Sadowski *et al.*, 2004). However, some researchers claim that cerebrovascular pathology acts independently of AD pathology, leading to earlier onset in patients with otherwise asymptomatic low-grade pathology (Snowdon *et al.*, 1997; Riekse *et al*., 2004).

1.3 Apolipoprotein E structure and function

Apolipoproteins are a group of proteins that have an important role in the lipid transport in the plasma as well as in the central nervous system: through binding to hydrophobic phospholipids, cholesterol and triglycerides, they promote they aqueous solubility. Apolipoprotein E (APOE) is the most extensively studied of the apolipoproteins, as it appears to perform multiple functions in the human body. It is a key component of the chylomicrons, very low density lipoprotein (VLDL) and high density lipoprotein (HDL) synthesised predominantly in the liver and the adipose tissue (Mahley *et al.*, 1984). In the nervous system APOE is synthesised predominantly by non-neuronal cell types, mainly astroglia and microglia, whereas the receptors for APOE are expressed by neurons (Zhang et al., 2013). The primary functions of APOE include transport and metabolism of cholesterol, other fatty acids and fat-soluble vitamins.

APOE is highly expressed in the brain as it an important lipid transport protein in the CSF. Upon peripheral nerve injury, it is produced in the large amounts to aid tissue repair by delivering lipids to recovering axons and to Schwann cells during remyelination (Huang and Mahley, 2014). APOE lipidation and metabolism are regulated predominantly by ATP-binding cassette A1 (ABCA1) cholesterol transporter, APOE receptors and APOE proteases (Liao *et al.,* 2017). Lipoprotein receptor-related protein 1 (LRP1) and LDLR are the key metabolic receptors for APOE (Kim *et al*., 2009); however, recent finding suggest that APOE may also bind to a microglial receptor TREM2 influencing TREM2-mediated microglial phagocytosis (Yeh *et al*., 2016).

APOE polypeptide consists of 299 amino acids and APOE4 differs from wildtype APOE3 by a single amino acid at position 112 (Cys_{112}- Arg) (Rall *et al.*, 1982). Changes in the amino acid sequence in APOE4 isoform can lead to structural modification and partial unfolding of the globule. Although, the partially folded protein was shown to have higher affinity of binding to lipid particles (e.g. membranes, lipoproteins) (Morrow *et al.*, 2002; Weers *et al.*, 2001), it tends to be more unstable and therefore susceptible to degradation by proteolysis *(*Hatters *et al.,* 2006*)*.

1.4 APOE gene and allele distribution

APOE gene is located on the long arm of chromosome 19 (q13.2) and is 3.7 kilo base pairs (kb) long. It has been shown to be highly conserved (~70% homology) within other mammalian species including rat, rabbit and canine (McLean *et al.,* 1983), indicating its high importance. Variants of the *APOE* gene E2, E3 and E4 are the result of the two missense single nucleotide polymorphisms (SNPs) rs429358 (T->C) and rs7412 (C->T), leading to amino acid changes (Cys -> Arg) at residues 112 and 158.

The allele frequencies vary between population and *APOE4* allele frequency tends to increase with latitude (Eisenberg *et al*., 2010). The most frequent genotype within human species is *APOE3*, especially within long-established agricultural economy populations, such as Mediterranean basin (85-89%) or East Asian population (82-87%) (Corbo and Scacchi, 1999), (G. Davies *et al.*, 2014) indicating an advantage of the APOE3 metabolic properties in a transition from the food collection to food production. Amongst the population where the food availability had been scarce until recently (e.g. aborigines of Australia or Lapps) the most common allele is *APOE4*. As the *APOE4* allele is associated with higher cholesterol absorption from the intestines as well as higher plasma cholesterol levels, carrying this allele may be beneficial under these circumstances (Corbo and Scacchi, 1999). It has been suggested that APOE2 isoform does not provide an advantage in any particular environmental conditions, as its distribution does not seem to exhibit any pattern amid different population (Corbo and Scacchi, 1999).

1.5 APOE role in AD pathogenesis

APOE can influence AD pathogenesis via a number of mechanisms. Recent studies indicate, that increased lipidation of the APOE, which is modulated by upregulation of ABCA1 by transcriptional and post-transcriptional mechanisms, inhibits Aβ deposition (Liao *et al*., 2017). Moreover, there are a number of pathways by which APOE facilitates Aβ clearance, such as enzymatic degradation, receptor-mediated blood-brain barrier (BBB) transport, and Aβ endocytosis by glial cells (Liao *et al.*,

2017). In addition to affecting Aβ deposition and clearance APOE may contribute to AD pathogenesis by affecting vascular function, neuroinflammation, metabolism, synaptic plasticity and transcriptional regulation (Liao *et al*., 2017).

A number of studies has previously investigated the levels of APOE mRNA or protein in the AD brain and the results are mixed. By most accounts, APOE protein levels were decreased in AD brains especially in the hippocampus and the frontal cortex regions and the effect was *APOE4* dependant. For instance, according to (Bertrand *et al.*, 1995; Beffert *et al.*, 1999) the APOE protein levels were lower in the hippocampus and frontal cortex of the AD tissue, which was also associated with increased Aβ levels (Beffert *et al.*, 1999). Other studies shown significantly higher APOE mRNA in AD brains compared to controls (Lambert *et al*., 1997; Yamagata *et al.,* 2001; Matsui *et al.*, 2007). A study by Mise *et al.* (2017) investigated *APOE* expression in the peripheral blood and they did not find any significant differences in the *APOE* mRNA levels between AD and controls.

1.6 APOE alleles as AD risk factor

Carrying at least one *APOE4* allele is the strongest known risk factor for late-onset AD, whereas the *APOE3* variant is believed to be neutral in the disease's risk, and the *APOE2* isoform may have protective properties. It has been estimated that 40% of all AD patients carry at least one ε4 allele, while homozygosity for *APOE4* allele increases the risk of AD from 20 to 90% as well as significantly decreasing the age of its onset (Corder *et al*., 1993). The ε4 allele effect on the AD risk is the greatest in the 60-70 year olds (Blacker *et al.*, 1997). *APOE4* allele has been previously associated with decreased APOE protein expression in mouse (Riddell *et al.*, 2008; Bray *et al.*, 2004) and human brains (Bray *et al.*, 2004). Interestingly, the mRNA expression was not significantly different between the 3 genotypes, suggesting that the mechanism that alters protein level is post-translational (Riddell *et al.*, 2008). Levels of cholesterol secreted from the astrocytes was also lower in the carriers od *APOE4* allele. Furthermore, the decreases in brain APOE protein levels were also associated with increased risk of developing AD, suggesting that the *APOE4* allele

may contribute to the AD risk by directly causing the APOE protein deficiency and thus impaired synaptic repair and Aβ clearance (Riddell *et al.*, 2008).
Decades of research have highlighted a number of functional differences among the three APOE isoforms, demonstrating their role in the AD pathology. The contribution of the APOE 4 isoform to the AD pathology can be divided into the Aβ dependent and that independent of the Aβ production. Numerous studies have investigated these mechanisms both *in vitro* and *in vivo* on the animal models as well as on the post mortem samples of the AD patients.

1.7 Aβ dependent mechanisms of the APOE isoforms on the AD pathology

The Aβ dependent pathways have been relatively well-studied as the Aβ deposition is the hallmark of the AD pathology. Different APOE isoforms can differentially affect the Aβ metabolism and thus aggravate the neuropathology and cognitive decline. Under certain conditions, APOE4 binds to and enhances the accumulation of Aβ. For instance, it has been shown that *in vitro* APOE3 and APOE4 that are unbound to lipids can form stable complexes with Aβ peptides rendering them resistant to degradation by dodecyl sulphate and guanidine hydrochloride and that formation of these complexes is faster and more efficient in case of the APOE4 isoform (Strittmatter *et al.*, 1994; Cho *et al.*, 2001). An *in vitro* study on transfected HEK cells, suggested that APOE3 binds to Aβ with higher affinity than APOE4 and therefore enhance clearance, preventing plaque deposition. However, the relevance of these results in the AD pathology should be further investigated, due to the differences of the APOE secreted by HEK cells and that in the CSF and the brain. Although, it has been also suggested that the interaction of APOE and soluble Aβ (sAβ) in physiological conditions is minimal and that the APOE influences sAβ metabolism not through direct binding, but other mechanisms, such as competition of sAβ and APOE molecules for the LRP1-dependent cellular uptake pathway in astrocytes. Furthermore, zinc- and copper-induced aggregation of the Aβ is increased in the presence of the APOE4 (Moir *et al.*, 1999). Moreover, according to (Castellano *et al.*, 2011; Deane *et al.*, 2008), APOE4 reduces the rate of Aβ clearance

in mice. Another study, conducted on APOE-deficient mice that expressed APP-V717F, demonstrated that APOE is actually necessary for the amyloid plaque formation in mice (Bales *et al.*, 1999). Similarly, (Kim *et al.*, 2011; Bien-Ly *et al.*, 2012) suggested that reduction of the APOE expression can lead to reduction of the Aβ levels and thus the rate of the plaque formation, as increasing the expression of the APOE3 and APOE4 in the hAPP or hAPP/PS1 transgenic mice, lead to increased accumulation of the amyloid plaques.

Numerous studies investigated effects of the APOE isoforms on the Aβ metabolism in the transgenic mice. Overall, the results indicate that mouse APOE enhances the Aβ accumulation (Bales *et al.*, 1999), whereas human APOE, in the absence of mouse APOE, reduces the plaques deposition (Holtzman *et al.*, 2000) and that APOE2 and APOE3 are more efficient at Aβ clearance than APOE4 (Holtzman *et al.*, 2000; Dodart *et al.*, 2005). One of the studies on the hAPP-V717I transgenic mice found that APOE4 expressed by neurons, unlike the one expressed by the glial cells, enhanced the amyloid plaque formation in the hippocampus and cortex (Van Dooren *et al.*, 2006). Although, in a different study, increased APOE4 expression in the astroglia and neurons did not have an effect on the Aβ levels (Lesuisse *et al.*, 2001). One of the suggested mechanisms of the APOE role in the Aβ clearance is astrocyte-mediated degradation and (Koistinaho *et al.*, 2004) suggested that different APOE isoforms differentially regulate astrocyte colocalisation and therefore modify their ability to clear the Aβ peptides.

Even though the majority of research suggests that APOE isoforms influence AD pathology mainly through regulation of the plaque formation, other studies indicate that there are mechanisms that are independent of the plaque formation (Buttini *et al.,* 2002; Raber *et al.*, 2000).

1.8 Aβ-independent effects of the APOE isoforms on the AD pathology

Although, APOE4 has a number of implications in the Aβ accumulation and toxic activity, it also has a role in the AD pathology independently of the Aβ. For instance, transgenic mice, which were induced to express human APOE4, instead of the endogenous mouse APOE, performed significantly worse in the cognitive testing

(water maze and the vertical exploratory behaviour) than the mice that were expressing human APOE3 or the wild type (Buttini *et al.*, 1999; Raber *et al.*, 1998). This effect was especially pronounced in the female mice, suggesting that the APOE4 effect could be gender-dependent. Morphology assessments of these transgenic mice, suggested that APOE3, but not APOE4 was protective against kainic acid-induced neurodegeneration that was apparent in the *APOE* knockout mice. Other studies have also suggested that APOE4 is linked to impaired memory and spatial learning in the transgenic mice (Andrews-Zwilling *et al.*, 2010; Leung *et al.*, 2012; Hartman *et al*., 2001). As there is no Aβ accumulation in these transgenic mouse models, these findings support the Aβ-independent role of APOE4 in the neurodegeneration. There are a few suggested mechanisms of the APOE protein fragments neurotoxicity. Examples of these would include induction of the Tau phosphorylation, cytoskeleton disruption and mitochondrial dysfunction. According to Chang *et al.* (2005), APOE4 is able to enter the cytoplasm by translocation facilitated by positively charged receptor-binding region, allowing the lipid-binding region to interfere with the mitochondria. *APOE* genotypes associate with the mitochondrial dysfunction, with *APOE4* carriers being more affected than *APOE3* carriers (Gibson et al., 2000). *APOE4* carriers (both AD and controls) have reduced glucose metabolism in the brain (Drzezga *et al.*, 2005; Hirono *et al.*, 2002; Mosconi *et al.*, 2005). This implies that APOE4 can have an effect on the AD development via dysregulation of mitochondrial function in the very early phases of the disease pathogenesis.

APOE3, unlike APOE4 binds to unphosphorylated tau, possibly preventing its phosphorylation (Strittmatter *et al.*, 1994). Transgenic mice expressing human APOE4 had significantly higher tau phosphorylation that was specific to neurons (Brecht *et al.*, 2004; Tesseur *et al.*, 2000a; Tesseur *et al.*, 2000b). By inducing tau phosphorylation, APOE can induce formation of neurofibrillary tangles and thus contribute to AD pathogenesis. Interestingly, removing tau protein, reduced the neurotoxic effect of the APOE4 in the transgenic mice (Andrews-Zwilling *et al.*, 2010). In a large GWAS study on the AD patients and controls, APOE4 allele was

associated with increased tau phosphorylation in CSF irrespectively of the Aβ levels (Cruchaga *et al.*, 2013).

As mentioned previously, APOE4 isoform is more sensitive to proteolysis than the APOE3 isoform and fragments produced by the degradation can impair the repair and remodelling leading to neurodegeneration (Huang, 2010); (Huang and Mahley, 2014). Significantly higher levels of the carboxyl-terminal-truncated APOE4 have been found in the Alzheimer's brains compared to controls and the levels were even higher in the carriers of two *APOE4* alleles (P. B. Jones *et al.*, 2011; Huang *et al.*, 2001; Harris *et al.*, 2003). The fragmented APOE4 were also deposited in the neurofibrillary tangles and amyloid plaques of the AD brains (P. B. Jones *et al.*, 2011; Huang *et al.*, 2001). *In vitro*, the APOE fragments that were expressed by the cells or added to the culture, stimulated the neurofibrillary tangle-like inclusions and thus were neurotoxic (Huang *et al.*, 2001; Ljungberg *et al.*, 2002).

APOE4 isoform also may also contribute to AD pathology by negatively affecting neuronal plasticity and neurite extension. *In vitro*, APOE4 was linked to neurite branching restriction and cytoskeleton disruption (Holtzman *et al.*, 1995; Nathan *et al.*, 1994). Similarly, in transgenic mouse models, APOE4 expression was related to disrupted cytoskeletal stability due to interference with microtubule formation and decreased β-tubulin polymerisation (Tesseur *et al.*, 2000a). Whereas APOE3, expressed by the mouse hippocampal neurons, enhanced neurite outgrowth (Sun *et al.*, 1998). Furthermore, in the cell cultures, APOE3 was more efficient at enhancing neurite extension than the APOE4 isoform. It has been suggested that APOE mediates neurite outgrowth via Erk pathway activation, which has been demonstrated in primary neuronal cell cultures.
APOE isoforms have also been shown to affect integrity of the BBB in mouse models (Bell *et al.*, 2012). Specifically, APOE4 increased the risk of the BBB damage due to activation of the inflammatory pathways.

Studies on transgenic mouse models demonstrated that APOE4 can be associated with hilar GABAergic neurons impairment and thus reduction in the hippocampal

neurogenesis, resulting in learning and memory difficulties. The role of APOE4 on the GABAergic mediated cognitive impairment have been also studied in humans. AD is associated with reduced GABA and somatostatin levels in the brain and CSF (Bareggi *et al.*, 1982; P. Davies *et al.*, 1980; Hardy *et al.*, 1987; Seidl *et al.*, 2001; Zimmer *et al.*, 1984) and this is more pronounced in the patients carrying at least one *APOE4* allele (Grouselle *et al.*, 1998). Reduction of GABA levels with age is the strongest known AD risk factor. Therefore these findings support the role of APOE4 isoform in the AD pathology via GABA regulation.

Despite all of the research conducted both *in vitro* and *in vivo* on animal models as well as human post mortem brains, the specific mechanisms through which different *APOE* alleles modify AD risk, remain incompletely understood. Moreover, carrying the *APOE4* allele alone is not enough to develop AD, nor is it essential for the disease development. It has been suggested that *APOE* alleles might display variation in the transcriptional regulation affecting the gene expression and therefore influencing the AD pathology.

1.9 TOMM40 gene in AD

TOMM40 gene is located on the long arm of the chromosome 19 (q13.32) closely adjacent to the *APOE* gene. It is 13.12 kb long and encodes for translocase of outer mitochondrial membrane 40 (TOMM40) protein. *TOMM40* is a polymorphic gene and numerous studies have investigated its role in the AD pathology.

Some polymorphisms in the *TOMM*40 gene have been implicated as a potential risk factor in AD (X. Y. Ma *et al.*, 2013), especially TOMM40'523 polyT length polymorphism, which has shown an association with the age of onset of AD (Roses *et al.*, 2010; Roses *et al.*, 2016). However, Mise *et al.* (2017) findings suggest that the TOMM40'523 polyT genotypes were not significantly different between AD and controls.

According to Mise *et al. (*2017), TOMM40 mRNA expression is significantly lower in the peripheral blood of the AD patients compared to controls and correlates with cognitive decline, which was measured using Mini-Mental State examination test. Overexpression of *TOMM40* mRNA has been suggested to be protective against mitochondrial dysfunction (by protecting against some mitotoxic effects of intracellular Aβ peptide) and thus neuronal damage (Zeitlow *et al.*, 2017). One study presented a hypothesis that mitochondrial dysfunction may be the first pathophysiological event in AD and that TOMM40 may have a protective role in this process (Ferencz *et al.*, 2012).

Due to the *TOMM40* gene location in the genome, preceding the *APOE* gene, it is thought to be in a linkage disequilibrium with the *APOE* gene (Yu *et al.*, 2007; Caselli *et al.*, 2012). One study suggested that certain *TOMM40* SNPs may be in a linkage with *APOE*4 allele and thus strongly associate with late onset AD (LOAD) (Yu *et al.*, 2007). However, after adjustment for confounders, only the *APOE4* association remained significant, suggesting that *TOMM40* only associates with the AD risk due to its linkage disequilibrium with the *APOE* gene (Yu *et al.*, 2007). Another study suggested that certain SNPs within *TOMM40* gene, *APOE* promoter and distal *APOE* enhancers influence CSF APOE protein levels (Bekris *et al.*, 2008). Therefore, it is imperative that the whole *TOMM40-APOE* locus rather than the *APOE* gene alone is investigated in the context of AD pathology.

1.10 Gene methylation in AD

Methylation is a process of adding a methyl group to a DNA sequence by an enzyme methyl transferase. It typically occurs at cytosine which is followed by guanine in a DNA sequence—a CpG site. A cluster of CpG sites in a given gene region is called a CpG island. Gene expression can be affected by methylation levels due to the methyl group blocking access for the transcription factors to the important regulatory regions. Transcription factor binding can up- or down-regulate gene expression, depending on its type. For instance, transcription factors may enhance or block binding of the RNA polymerase to DNA; alternatively, they can affect histone acetylation by catalysing histone acetyltransferase (HAT) or histone deacetylase (HDAC). They can also catalyse the reactions indirectly by facilitating binding of the coactivator or corepressor protein to the complex. Promoter and enhancer regions methylation would typically have the highest influence on the gene expression. In majority of cases, hypermethylation of the promoter or enhancer would lead to decreased gene expression (Bae *et al.*, 2016). There are, however, cases where highly regulatory regions were associated with enhanced expression. For instance, *TREM2* (a gene that has been associated with AD) promoter hypermethylation is associated with enhanced gene expression (Smith *et al.*, 2016).

Numerous environmental factors such as diseases can affect gene methylation. Ageing and age-related diseases associate with methylation levels both in humans (Siegmund *et al.*, 2007; Xiao *et al.*, 2015; Peters *et al.*, 2015; Slieker *et al.*, 2016; Bollati *et al.*, 2009; Reynolds *et al.*, 2015) and mouse models (Chouliaras *et al.*, 2012; Maegawa *et al.*, 2010). DNA is differently methylated in AD patients and in mouse models (Chouliaras *et al.*, 2012; Coppieters *et al.*, 2014; Sanchez-Mut *et al.*, 2013), which may indicate that DNA methylation could influence the AD pathogenesis. Due to the strong association of ageing and the methylation levels, it has been suggested that gene methylation may have crucial role in AD disease development as the age of onset is typically over 65 years.

1.11 *TOMM40-APOE* locus methylation in association with AD

Methylation of the APOE gene has been studied relatively well, especially the promoter region, which is hypomethylated as well as the exon 4 CpG island, which, on the contrary, is highly methylated (Y. Ma *et al.*, 2015). APOE methylation may be influenced by the genotype, especially considering that SNPs responsible for the three APOE alleles may either disrupt or create a CpG site. Indeed, there are certain studies that show significant association of the *APOE* methylation levels with the genotype (Ma *et al.*, 2015). Furthermore, *APOE* methylation levels have been associated with plasma total cholesterol levels (Ma *et al.*, 2015).

APOE gene methylation has been previously shown to influence APOE protein level and thus correlate with AD pathology. For example Foraker *et al.* (2015) and Tulloch *et al.* (2018a) found that the APOE gene displayed significantly lower methylation in the frontal lobe and the hippocampus of the AD patients compared to the controls. Interestingly they found no methylation difference in the cerebellum, which is known to be less affected by AD than the two other aforementioned regions (Foraker *et al.*, 2015). An exploratory study on the small number of brain (n = 12) and blood samples (n = 67) suggested that methylation of the *TOMM40-APOE-APOC2* gene region may associate with AD pathology via regulating *TOMM40* and *APOE* expression (Shao *et al.*, 2018). Moreover, they suggested that the methylation levels of the locus differed significantly between the blood and brain samples (Shao *et al.,* 2018). Similarly, a recent study on Swedish twins found that methylation of the *APOE* promoter region (including the intergenic region between *TOMM40* and *APOE* gene) was increased in the AD peripheral blood and that the effect was independent of the APOE genotype (Karlsson *et al.*, 2018). As majority of previous studies only investigated the effect of the *APOE* region methylation on AD in a small number of samples, it is still not fully understood how methylation of the *TOMM40-APOE* locus in the brain relates to AD neuropathology in a larger number of samples characterised for longitudinal cognitive decline.

1.12 Aims and objectives.

1.12.1 Aims

To investigate whether the *TOMM40-APOE* locus is differentially methylated in the AD brains and whether their methylation level correlates to the APOE protein levels in the brain.

1.12.2 Objectives

1. Investigate a collection of AD and healthy prefrontal cortex samples for levels of APOE protein using an ELISA and relate this to AD neuropathology (i.e. Braak stage, Thal and CERAD score).

2. Investigate DNA methylation differences at the *APOE* exon 4, *TOMM40* promoter region and the intergenic region, using bisulphite pyrosequencing, and relate to *APOE* alleles and APOE protein levels in the brain.

3. Investigate the localization of the APOE protein in the prefrontal cortex of AD brains compared to the healthy brains using the immunohistochemistry (IHC) staining with anti-APOE antibody.

1.13 Hypothesis

APOE methylation is increased in AD, leading to decreased *APOE* gene expression and thus reduced levels of APOE protein in the brain. The methylation of CpG sites in the surrounding regulatory regions *TOMM40* gene and the intergenic region also influence the APOE protein expression.

By regulating APOE protein expression, the methylation levels associate with AD pathology and cognitive performance.

The *APOE* alleles differentially regulate AD pathology partially via affecting methylation levels.

2. Materials and methods.

2.1 Study material

The prefrontal cortex brain samples that were used for the analysis in this study were obtained from Manchester Brain Bank. The participants included 46 females and 21 males (mean age of death 87.5, SD=6.1) of Caucasian population from Manchester. The participants were previously tested for fluid intelligence, vocabulary, speed and memory. Both cross-sectional and longitudinal biennial cognitive assessment results were included in the analysis. The method of the cognitive assessment was previously described (Rabbitt *et al.*, 2004).

Ethical approval has been granted for the original study where the samples have been obtained from and no further ethical approval was necessary for the present study. All subjects have provided a written consent to donate their post-mortem samples to the brain bank for research purposes.

Risk assessments and COSHH form have been completed prior to the commencement of this study and all of the necessary precautions have been undertaken to ensure safety and security in the laboratory environment.

2.2 Brain pathology assessment

The brain samples were previously assessed and staged by experienced pathologists at the brain bank using the 'ABC score' recommended by National Institute on Aging–Alzheimer's Association (Hyman *et al.*, 2012), involving 3 scores: Thal—Aβ plaque score (Thal *et al.*, 2002) Braak–neurofibrillary tangles stage (Braak and Braak, 1991) and CERAD—neuritic plaque score (Mirra *et al.,* 1991). AD and controls were defined on the basis of these pathological scores. AD was defined as scores of 3 or grater in Braak, B or higher in CERAD and 2 or higher in Thal.

2.3 APOE protein quantification

The protein lysate used for the APOE protein quantification was previously extracted from the brain tissue using RIPA buffer (Sigma) and 1x protease inhibitor cocktail (Sigma). The procedure has been described previously in detail (Bradburn *et al.*, 2018).

The levels of the APOE protein in the brain were measured using Abcam Apolipoprotein E Human ELISA kit according to the manufacturer's instructions. The absorbances at 450 and 570 nm were measured using Synergy HT microplate reader. The raw data was then analysed using Microsoft Excel 2016 and Prism Graphpad (version 7.0a). To enable estimation of the APOE protein concentration, 7 standards of known concentrations were included on the plate and their concentration vs absorbance were plotted as a hyperbola standard curve using Prism Graphpad (version 7.0a). The unknown concentrations of the samples were then interpolated onto the curve using the known absorbance. The raw values were then standardised for the total protein concentration in each sample.

2.4 DNA extraction

Genomic DNA was extracted from the prefrontal cortex samples of the brain using Bioline Isolate II genomic DNA Kit (Bioline, UK), according to the manufacturer's instructions. The concentration and purity were measured using NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). Absorbance ratios at 260/280 and 260/230 were used as the indicators of the DNA purity, to ensure the DNA was free of protein and salt contamination. DNA solutions were stored at -80C until further analysis.

2.5 Genotyping

Genotyping of the APOE rs7412 and rs429358 was done previously and described together with the quality control procedures by Davies *et al.* (2014). Briefly, the two *APOE* SNPs were genotyped using the Sequenom MassARRAY iPLEX platform (G. Davies *et al.*, 2014). Table 1 shows how the rs429358 and rs7412 genotypes represent E2/E3/E4 alleles.

Table 1 Genotypes associated with APOE alleles

Alleles	Rs429358 genotype	Rs7412 genotype
E2/E2	TT	TT
E2/E3	TT	TC
E2/E4	TC	TC
E3/E3	TT	CC
E3/E4	TC	CC
E4/E4	CC	CC

2.6 Methylation analysis

2.6.1 Bisulphite treatment

The DNA samples (250 ng) were bisulphite converted with the use of Qiagen EpiTect Fast DNA Bisulphite Kit (Qiagen, Hilden, Germany). Prior to the conversion, the DNA samples were diluted to achieve an equal concentration of 12.5 ng/µL. The samples were then mixed with bisulphite solution and DNA protect buffer and incubated in the Eppendorf Mastercycler thermal cycler, using thermal conditions specified by the kit's manufacturer (shown in Table 2). The converted DNA was then treated with DNA binding buffer and placed in the spin columns to perform wash steps for desulphonation and purification. A volume of 30 µL was eluted (2 x 15µL) and stored at -20° C until amplification.

Table 2 Bisulphite conversion thermal cycle conditions.

Step	Description	Time	Temperature [°C]
1	Denaturation	5 minutes	95
2	Incubation	10 minutes	60
3	Denaturation	5 minutes	95
4	Incubation	10 minutes	60
5	Hold	indefinite	20

2.6.2 Target regions analysed and primers used

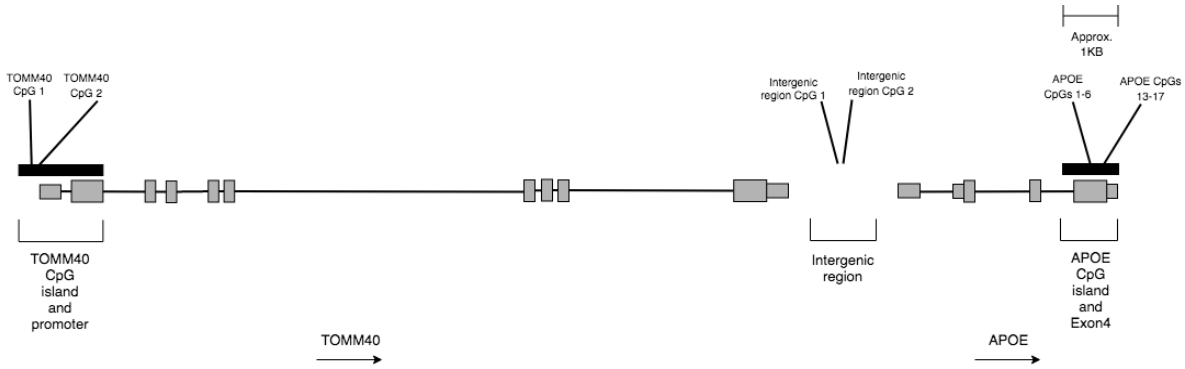

Figure 1 Genomic map of TOMM40- APOE locus showing approximate locations of the CpG sites investigated in this study. Black bars represent CpG islands. Grey bars represent gene exons. Transcription of the genes occurs from left to right as shown by arrows. Schematic representation - the scale only approximately represents the length of genes.

The three regions of interest, *TOMM40* promoter region, *APOE* exon 4 and the intergenic region were identified using previous publications (Shao *et al.*, 2018; Foraker *et al.*, 2015) and Genome browser (University of California Santa Cruz) was used to get their sequences. Figure 1 show the approximate location of the investigated CpGs in the *TOMM40-APOE* locus. The primers that were used for DNA amplification and pyrosequencing were designed using Pyromark Assay Design SW 2.0 (Qiagen, Hilden, Germany) and manufactured by Invitrogen by Thermo Fisher Scientific. The forward, reverse and sequencing primers and the sequence analysed for each region are shown in table 3.

Table 3 Sequences in TOMM40 promoter APOE exon 4 and the intergenic region analysed in this study and primers used for their amplification and sequencing

Gene location	Primers; Forward, reverse, sequencing * Biotin tagged	Sequence analysed (CpGs numbered) 5'-3'
APOE exon 4	F: ATGAAGGAGTTGAAGGTTTATAAAT R: AACCCCCACCTAATACACTACCAA * S1: GGGTAAGGTTGTTTAAGGA S2: GGGTGAGTTTAGTTTTTTATTTG	1.GTTGTAGGYGGYGTAGGTTYGGTTGGGYGYGGATATGGAG GAYGTGYGYGGTYGTTTGGTGTAGTATYGYGGYGAG 2. YGTAAGTTGY GTAAGYGGTT TTTTYGYGAT GTYGATGATT TGTAGAAGYG TTTGGTAGTG TATTAGGTYG
TOMM40	F: GTTTTAGGGTAGAAGGATAGGTAAAGG R: AACACTCTAAAAAAAACCAACACTT * S: TTTGGTTAGGAGTAGT	YGATTTAAAA TYGGAAGTGT TGGTTTTTTT TAGAGTGTTT
Intergenic region	F: TTGGGATTTATAATAGGGTTTAGGAAAGTG R: CCTCTCTAAACATCAAATTCCTTTACT * S: AGGGTTTAGGAAAGTGATA	GYGTTTGAGY GTTTATTGTG GTTTGTTTAT TGTTAGTTTT AATATAGGAT YGTTGTGTGT TAGGGTTGTT TTTTATGTTT AATATAYGTT AGTTTGTTAT TAAATATATT YGTGTYGTTG TTTTTTTAGT TTGATGAGTA AAGGAATTTG ATGTTTAGAG AGGATAAGTT ATTTGTTTAA GGTTATATAG TTGGTAATTG GTAGAGTTAG

2.6.3 Polymerase chain reaction (PCR)

A hot start PCR was performed using the forward and reverse primers shown in table 4. The master mix was prepared using 12.5 µL MyTaq HS (Bioline), which contains DNA polymerase, dNTPs, $MgCL_2$ and enhancers, 0.5 µL forward primer, 0.5 µL reverse primer, 2.5 µL coral load dye and 7 µL molecular grade water. The reactions containing 2 µL of the bisulphite converted template DNA and 23 µL of the master mix were set up in the sterile 200 µL PCR tubes, spun down using a centrifuge and amplified using Eppendorf Mastercycler thermal cycler. All of the preparations were carried out on ice to avoid unspecific binding and the preparation of the master mix was conducted under a PCR hood to avoid foreign DNA contamination. Initially, the thermal cycling conditions recommended by the MyTaq manufacturer were used. However, the gel electrophoresis and pyrosequencing results shown that these conditions were not satisfactory. Therefore, the primer annealing temperatures were optimized using a gradient. Ultimately, the annealing temperature of 58°C was used for all of the amplification of all three regions (see results).

Table 4 DNA amplification PCR thermal cycling conditions

Step	Description	Temperature (°C)	Time	Number of cycles per step
1	Hot Start (DNA Polymerase activation)	95	5 minutes	1 cycle
2	Denaturation	95	30 seconds	50 cycles
	Annealing	56	30 seconds	
	Extension	72	30 seconds	
3	Hold	4	indefinite	1 cycle

2.6.4 Agarose gel electrophoresis

Gels were made using 2% of agarose mixed with 1 x tris-borate-EDTA (TBE) buffer and Midori Green Advance DNA stain (NIPPON Genetics Europe) in the proportion of 5 µL of stain per 100 ml of gel. The PCR products and 50 bp DNA hyperladder (Bioline) were loaded onto the gel and electrophoresed in the tanks containing 1x TBE buffer at 90 V for 30-60 minutes. Coral load dye was added to the PCR reactions prior to their amplification. The gel images were acquired using Odyssey® Fc imaging system (LI-COR Biotechnology, UK).

2.6.5 Bisulphite pyrosequencing

DNA methylation analysis of the *APOE, TOMM40* and the intergenic region was carried out using the bisulphite pyrosequencing method on the Pyromark Q24 system (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The sequencing master mix contained Streptavidin Sepharose High Performance beads (GE Healthcare Biosciences), Pyromark binding buffer and molecular grade water in the ratio of 1:40:19 respectively. 60 µL of the master mix was combined with 20 µL of the PCR product on the 24-well plate and shaken at 1400 RPM for 10 minutes at room temperature using the TS-100 thermo-shaker (BioSan). Samples were then placed on to the Pyromark workstation and processed with the vacuum filtered probes for 15 seconds to ensure all of the liquid was aspirated and the beads containing the DNA template were captured in the probe's filter. The probe was then flushed for 5 seconds in 70% ethanol, 5 seconds in denaturation buffer and 10 seconds in wash buffer to ensure all the DNA fragments that were unbound to the beads were washed away. The probe was subsequently placed onto the 24-well sequencing plate containing the sequencing primers diluted to 0.3 µM concentration with Pyromark annealing buffer, ensuring the vacuum was switched off, and shaken to enable the beads to be released into the wells. The samples were then incubated at 80° C for 2 minutes on a heating block (DB-2D, Dri-Block®, Techne). The sequencing plate was then placed in the Pyromark Q24 sequencer together with a cartridge containing enzyme mix, substrate mix and the deoxy nucleotide triphosphates (dNTPs) in the proportions according to the pre run information for the specific run on the PyroMark Q24 Advanced software.

2.7 Immunohistochemistry staining

The prefrontal cortex samples (3 control and 3 AD) were cut into sections using the cryostat at -18° C and stored at -80° C until the analysis. The slides were air dried after thawing, re-hydrated with 0.05% PBS-T for 10 minutes and then blocked for 30 minutes with 4% goat serum in PBS-T to prevent unspecific binding. The sections were then incubated for 24 hrs at room temperature with rabbit APOE polyclonal antibody (ProSci, Poway, USA) at concentration of 20 µg/mL and Novocastra^{TM} Glial Fibrillary Acidic Protein (GFAP) mouse monoclonal antibody (Leica Biosystems, Newcastle, UK) at concentration 0.14 ug/mL (both concentrations recommended by the manufacturers for the purpose) diluted in the blocking buffer (4% goat serum). Following incubation the slides were rinsed with PBS-T for 5 minutes and incubated for 30 minutes with two secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 568 goat anti-mouse IgG, both diluted 1 in 200 in blocking buffer. The slides were subsequently mounted using Vectashield antifade mounting medium with DAPI (Vector Laboratories, Peterborough, UK) and coverslipped. The slides were then wrapped in aluminium foil to avoid fading due to light exposure and stored at 4° C. The stained slides were then viewed and photographed under fluorescent microscope at magnification x200 and x630.

2.8 Statistical analysis

All statistical analyses were performed in the SPSS statistics (version 25, IBM statistics). The significance threshold was accepted at $p<0.05$. In case of the Hardy-Weinberg equilibrium, the $P> 0.01$ was accepted due to the small sample size and low minor allele frequency. The continuous data was tested for normal distribution using Kolmogorov- Smirnoff test. Data are presented as mean $\pm$ standard deviation (SD) if normally distributed or median and 25^{th}- 75^{th} percentiles if not normally distributed, if otherwise stated. Square root transformation was performed on APOE protein levels data and arcsine transformation on some of the CpG methylation data to achieve normality. Continuous Pearson's Chi-squared goodness of fit test was used to determine if the alleles were in Hardy-Weinberg equilibrium. Continuous data were tested using t-test to determine differences between AD and

controls. Chi-square tests were performed to determine distribution differences between nominal data. T- test (or a Mann Witney-U test for not normally distributed data) was used to determine differences in APOE protein levels and methylation levels between the genders. The methylation levels as well as the protein level were also tested using two-way ANOVA to determine possible genotype (*APOE4* carriers vs non *APOE4* carriers) and disease (AD vs controls) effects as well as disease x genotype interactions. To determine differences in the cognitive test between AD and controls, Mann-Whitney U test was performed. Spearman correlation test was performed to determine association of the ApoE protein level and the methylation status with the AD pathology scores (Thal staging for amyloid deposition, Braak staging for neurofibrillary tangles and CERAD staging for neuritic plaques). To adjust for confounding factors, ordinal regression model was performed on factors that associated significantly. Pathology stages were set as dependent variable and APOE protein level as well as gene methylation were set as independent variables together with other potential confounders including age at death and *APOE4* allele, as both of these could have an effect on the brain pathology. To determine association of APOE protein level and methylation status with cognitive measures, Pearson correlation was performed initially, followed by linear regression. Cognitive measure was set as dependent variable and APOE protein level, methylation status, gender, age at cognitive testing and *APOE4* presence were set as independent variables to determine, whether the correlation remained significant after adjustment for confounding factors.

3. Results

3.1 PCR optimization

To improve the efficiency of the bisulphite pyrosequencing, the PCR reactions needed to be optimised to ensure sufficient yield and avoid unspecific binding, which could influence quality of the product. The quality and yield of the PCR product was tested using agarose gel electrophoresis. Initially, when the thermal conditions recommended by the manufacturer of the master mix were used, the bands on the gel image were faint, suggesting low concentration of the product. When the PCR product was tested on the pyrosequencer, the signal was very low and the sequencing failed. Firstly, the reactions were optimized by increasing the annealing temperature from the initial 56° C to 58° C. Figure 2 shows the PCR performed with a gradient of annealing temperatures 55 +/- 3° C. In case of *TOMM40* and the intergenic region, the annealing temperature of 58° C was optimal and produced enough high-quality product, which then could be used for bisulphite sequencing. However, in case of the APOE region, the annealing temperature adjustment on its own did not give sufficient yield to produce high enough signal on the pyrosequencer. Therefore, both forward and reverse primers were redesigned and tested in different combinations. The new sets of primers were tested for different annealing temperatures, to determine which will result in optimal quality and yield of the product. The results gel image is shown in figure 3 and 4. The optimal annealing temperature that was used for the three regions was found to be 58° C. *TOMM40* and the intergenic region PCR did not require any further optimization.

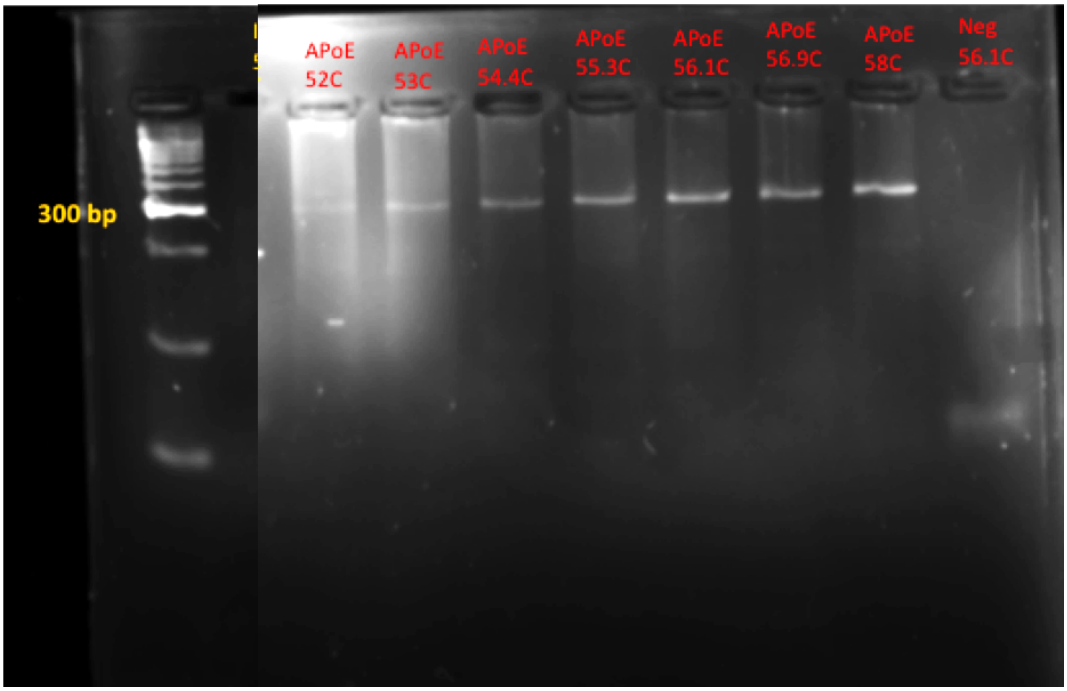

Figure 2 PCR optimization for the APOE exon 4 DNA region using the annealing temperature gradient starting at 55°C +/- 3°C. (The image was cropped to remove bands from another analysis that was not relevant to this study)

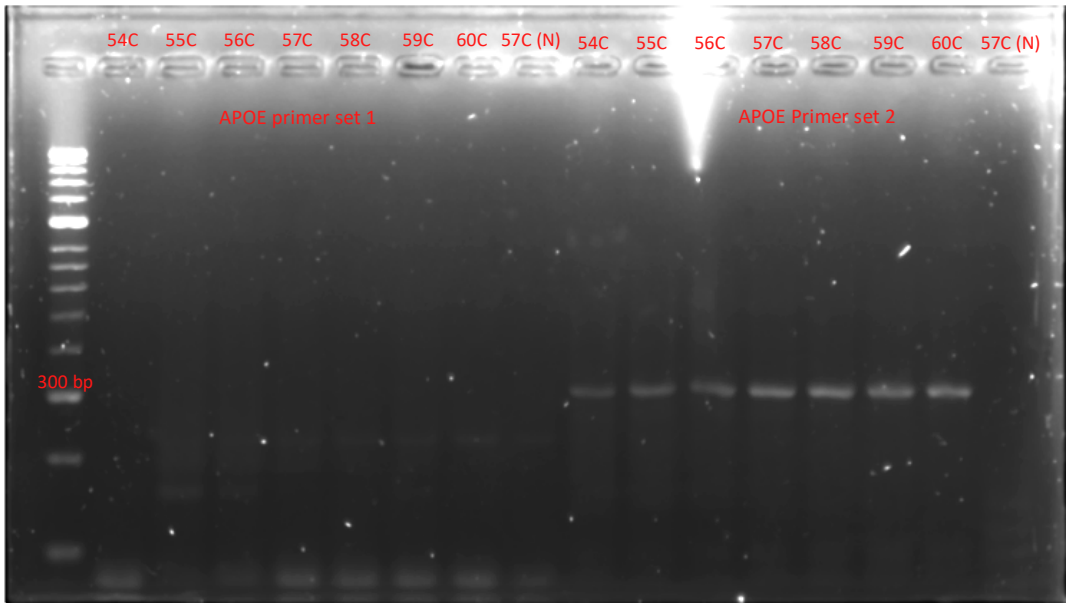

Figure 3 Gel image showing results of a PCR using two different APOE primer combinations at a gradient starting at 57°C +/- 3°C. Lanes 9 and 17 show the negatives. Ultimately second primer combination at annealing temperature of 58°C was used.

3.2 Pyrosequencing assay optimization and troubleshooting

The major issue with the pyrosequencing method that was encountered, was the signal strength. The first line of troubleshooting was PCR optimization discussed in the previous paragraph. When the maximum yield of the PCR product was achieved, and the sequencing signal was still insufficient, the volume of the PCR product added to the sequencing reaction was increased from 10 µL to 20 µL and the volume of water was reduced accordingly to ensure the concentration of other reagents remained constant. In case of the *TOMM40* and the intergenic region, sequences (which were relatively short), increasing the amount of PCR product added to the reaction resolved the problem of low signal strength and did not need to be further optimised. In case of the *APOE* region with a significantly longer sequence used initially, the sequencing primers were re-designed and two shorter sequences were analysed separately. This greatly increased the signal strength. However, the volume of the PCR product added to the sequencing reaction needed to be further increased to 25 µL. Despite all these steps being undertaken, a number of samples did not give satisfying results for the APOE region methylation, specifically 21% of the samples for the sequencing primer 1 and 13% for the sequencing primer 2. Due to the time constrains of the project, the assays could not be further optimised and only the results that were of acceptable quality were included in the analysis.

3.3 *APOE* Genotypes

Genotypes of all of the 67 is show in table 5 (G. Davies *et al.*, 2014). The Chi-squared goodness of fit analysis revealed that both rs429358 and rs7412 alleles were in Hardy Weinberg equilibrium (p = 0.988 and p=0.034 respectively).

Table 5 Genotype frequencies of APOE rs429358 and rs7412.

	Genotype frequency [n(%)]		
SNP	**CC**	**CT**	**TT**
Rs429358	**2 (3.0)**	**20 (29.8)**	**45 (67.2)**
Rs7412	**57 (85.1)**	**8 (11.9)**	**2 (3.0)**

3.4 Cohort characteristics

Table 6 shows the participants characteristics stratified by disease status. Although all of the samples have had the pathological assessments performed at the centre (including Thal, Braak and CERAD), it was difficult to clearly assign some of the samples to AD or control group, due to discrepancies between different scoring systems, which led to difficulty in distinguishing between age changes and early signs of AD. Moreover, some of the samples would display pathology characteristic for diseases such as dementia with Lewy bodies (DLB) or cerebrovascular disease together with AD pathology, which could have affected the results. Therefore, to avoid biased results, only 62 (32 controls and 30 AD) were used in the analysis of differences between healthy aged and AD brains. The data were normally distributed and presented as mean (SD). The gender distribution was similar in both groups, with females making up 67.7% of the cohort. The overall mean age of death was 87.43 years and it was not significantly different between AD patients and controls (p=0.619). Similarly, the post mortem delay and whole brain weight did not differ between the two groups (p=0873 and p=0276 respectively). The number of *APOE4* carriers was significantly higher in the AD (46.7%) group than in controls (18.8%) (p=0.019). As expected, AD patients had significantly higher scores in all three pathological assessments (Thal, Braak and CERAD) than the controls ($p<0.001$), as they were assigned into groups based on these scores.

Table 6 Cohort characteristics. (Data presented as mean (standard deviation) unless otherwise stated. P- value was calculated using t-test unless otherwise stated.)

Variable	Controls (n=32)	AD (n=30)	p-value
Age at death [years]	87.03 (5.87)	87.83 (6.70)	0.619
Females [n(%)]	21 (65.6)	21 (70.0)	0.713 ($X^2(1)=0.136$)[d]
Post mortem delay [hours][a]	76.81 (47.97)	74.96 (37.63)	0.873
Whole brain weight [g][b]	1170.64 (118.71)	1214.33 (128.40)	0.276
ApoE4 carrier [n(%)]	6 (18.8)	14 (46.7)	0.019 ($X^2(1)=5.52$)
Thal stage (amyloid deposition) [n(%)]			<0.001 ($X^2(5)=49.95$)[d]
0	16 (50.0)	0	
1	11 (34.4)	0	
2	3 (9.4)	3 (10.0)	
3	2 (6.3)	13 (43.3)	
4	0	7 (23.3)	
5	0	7 (23.3)	
Braak stage (neurofibrillary tangles) [n(%)]			<0.001 ($X^2(3)=57.14$)[d]
0	4 (12.9)	0	
1	27 (87.1)	1 (3.3)	
2	0	21 (70.0)	
3	0	8 (26.7)	
CERAD score (neuritic plaques) [n(%)]			<0.001 ($X^2(3)=54.48$)[d]
0	17 (53.1)	0	
A	14 (43.8)	1 (3.3)	
B	1 (3.1)	17 (56.7)	
C	0	12 (40.0)	

[a] data available in controls n=30 and AD n=25
[b] data available in control n=22 and AD n=18
[d] P-value calculated using Pearson Chi-square test

3.5 Cognitive scores and their correlation to Braak, CERAD and Thal

All the cognitive scores data used for the analysis in this study were obtained previously by Rabbitt *et al.* (2004). Cognitive scores were tested using a t-test (or a non-parametric equivalent for non-normally distributed data) to determine differences between the gender groups. There were no significant differences between males and females in any of the cognitive scores. Figure 4 shows longitudinal fluid intelligence score stratified by gender. To determine the differences in the prior cognitive measures in AD and controls, t-test for normal data and Mann Witney-U test for non-normal data were performed, and revealed that lower cross-sectional and longitudinal speed were associated with AD (p=0.022 and p=0.008 respectively). To determine how prior cognitive scores are related to amyloid plaques, neuritic plaques and neurofibrillary tangles development at an older age, Spearman rank correlation between cognitive measures and Thal, CERAD and Braak scores was performed (Table 7). Test of correlation revealed that longitudinal fluid intelligence was significantly negatively associated with Thal (ρ=-0.371, P=0.003) and CERAD (ρ=-0.259, p=0.042) scores. To adjust for potential confounders, ordinal regression test was performed. In the first model, Thal or CERAD were set as dependent variables and gender was set as an independent variable, whereas age at death and longitudinal fluid intelligence were selected as covariates. Ordinal regression revealed that after adjustment for sex and age at death, longitudinal fluid intelligence remained significantly associated with Thal score (p=0.013), however, the association with CERAD was no longer significant (p=0.103). A second model was introduced for the Thal association with longitudinal fluid intelligence, where APOE4 allele presence was selected as an independent variable in addition to the variables from the first model. Even though APOE4 allele presence significantly associated with Thal score (P=0.001), the association of longitudinal fluid intelligence remained significant after testing for this confounding factor (P=0.017). When the gender was removed from the model, as it did not associate significantly with the cognitive scores, the P value was 0.012.

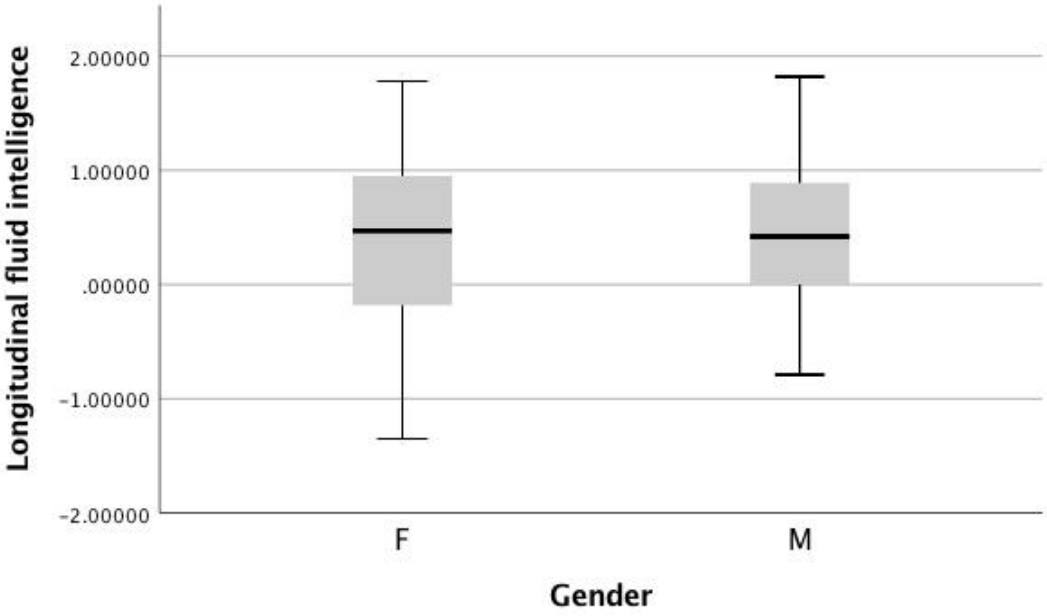

Figure 4 Longitudinal fluid intelligence score stratified by gender.

Table 7 Spearman rank correlation between the cognitive measures and AD pathology scores. The test was performed in both AD and controls collectively.

Variable	Thal score	CERAD score	Braak score
Cross sectional fluid intelligence	-0.198	-0.057	-0.037
Cross sectional vocabulary	0.053	0.068	0.048
Cross sectional speed	-0.185	-0.204	-0.196
Cross sectional memory	-0.018	-0.053	-0.053
Longitudinal fluid intelligence	-0.371**	-0.259	-0.205
Longitudinal vocabulary	-0.012	0.023	-0.008
Longitudinal speed	-0.183	-0.230	-0.224
Longitudinal memory	0.016	-0.034	-0.007

**Correlation is significant at the 0.01 level

3.6 APOE protein levels in the prefrontal cortex

The APOE protein levels were successfully measured in 62 as three samples did not have enough of the protein lysate for the analysis and in case of the two remaining samples the protein level was below the level of detection of the used method. The data were not normally distributed and therefore needed to be transformed using square root calculation to achieve normality and it is presented as square root of the original unit. Figure 5 shows APOE protein levels stratified by gender, values were not significantly different between males and females (P=0.602)

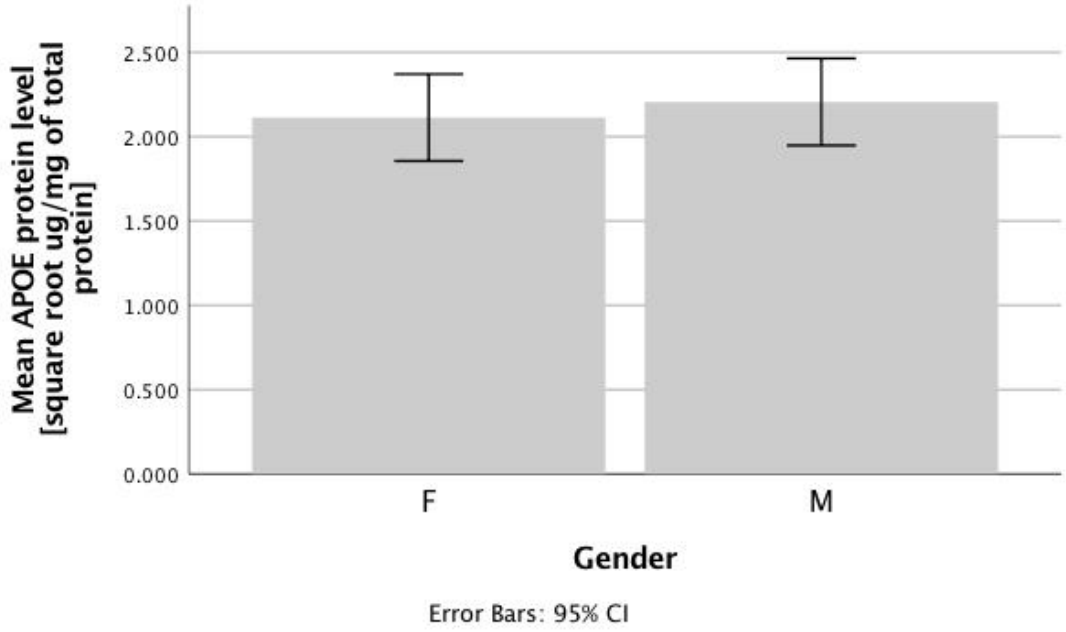

Figure 5 APOE protein levels in the prefrontal cortex stratified by gender.

3.7 APOE protein levels stratified by the disease status and *APOE4* allele presence

To investigate if the APOE protein levels differed in the AD compared to controls as well as between the APOE genotypes, two way ANOVA was performed and the results are shown in table 8. The test revealed that there were no significant differences in the brain APOE protein levels between the APOE4 carriers vs APOE4 non carriers in either AD or controls.

Table 8 APOE protein levels stratified by the disease status and APOE4 allele presence/absence.

Variable	AD		Control		Effects (P-value)		
	APOE4 carrier	APOE4 non-carrier	APOE4 carrier	APOE4 non-carrier	Disease group	APOE Genotype	APOE Genotype x Disease group
ApoE protein level [√ug/mg protein]	**2.01 (0.96)**	**2.09 (0.61)**	**1.78 (0.75)**	**2.41 (0.66)**	**0.566**	**0.069**	**0.193**

3.8 Correlation of the APOE protein levels with the AD pathology scores.

Spearman rank correlation test was performed to test whether the APOE protein levels associate with AD pathology scores. Neither Thal, Braak nor CERAD score significantly associated with the APOE protein levels (table 9).

Table 9 Spearman correlation of the APOE protein levels and the AD pathology scores- Thal, Braak and CERAD.

Variable	Thal score	Braak score	CERAD score
ApoE protein level	**-0.240**	**-0.183**	**-0.147**

3.9 APOE protein levels and the cognitive performance.

Pearson correlation was performed to test if the APOE protein levels were associated with previous cognitive scores, including fluid intelligence, vocabulary, speed and memory, both cross sectional as well as longitudinal. The result suggest that APOE protein levels in the prefrontal cortex are significantly associated with longitudinal fluid intelligence (R=0.265, P<0.05). No other cognitive score was significantly associated with APOE protein level (Table 10).

Table 10 Pearson correlation of the APOE protein levels and cognitive scores

Variable	CS fluid intelligence	CS vocabulary	CS speed	CS memory	L intelligence	L vocabulary	L speed	L memory
APOE protein level [Vug/mg protein]	0.177	0.190	0.216	-0.203	0.265*	0.217	0.188	-0.107

*correlation was significant at P < 0.05. CS-cross sectional, L-longitudinal.

Linear regression analysis was performed to further investigate the association between longitudinal fluid intelligence and the APOE protein levels. To account for other potential confounders, age at cognitive testing and *APOE4* allele presence were also included in the model as independent variables alongside the *APOE* protein level, while longitudinal fluid intelligence was set as dependent variable. After adjusting for confounding factors, association of the longitudinal fluid intelligence with the APOE protein level remained significant (B=0.279, P=0.049).

3.10 *TOMM40* promoter, intergenic region and *APOE* exon 4 methylation

For quality control purposes, at least eleven samples per assay were randomly selected and their methylation levels were measured twice on separate occasions. The coefficient of variation (CV = SD / mean x 100%) was calculated for each CpG site in a particular sample and the mean of all of the CVs for a given CpG site was calculated. For APOE and the IR CpGs only the CpG sites with mean CV below or equal 20% were included in the analysis. In case of the TOMM40 region, where the methylation levels were very low (mean=2.0, SD=1.1), despite the relatively high mean CV value for both CpG sites (24%), both CpG sites tested were included in the analysis. The CV values were higher in case of the hypomethylated region due to the limited specificity of the assay (up to 1%), therefore the variation between the repeated values could in fact be much lower than indicated. Eleven CpG sites in the APOE exon 4 region surrounding the rs429358 and rs7412 polymorphisms, two TOMM40 promoter region CpG sites and two intergenic region (IR) (located between the two genes) CpG sites were successfully assessed for methylation levels. The APOE exon 4 region and the IR highly methylated (APOE mean=63.3, SD=8.6; IR mean=57.5, SD=7.5). Whereas the TOMM40 promoter region was hypomethylated (mean=2.0, SD=1.1). Methylation levels were not significantly different between males and females at any CpG site tested (Figures 6-9) .

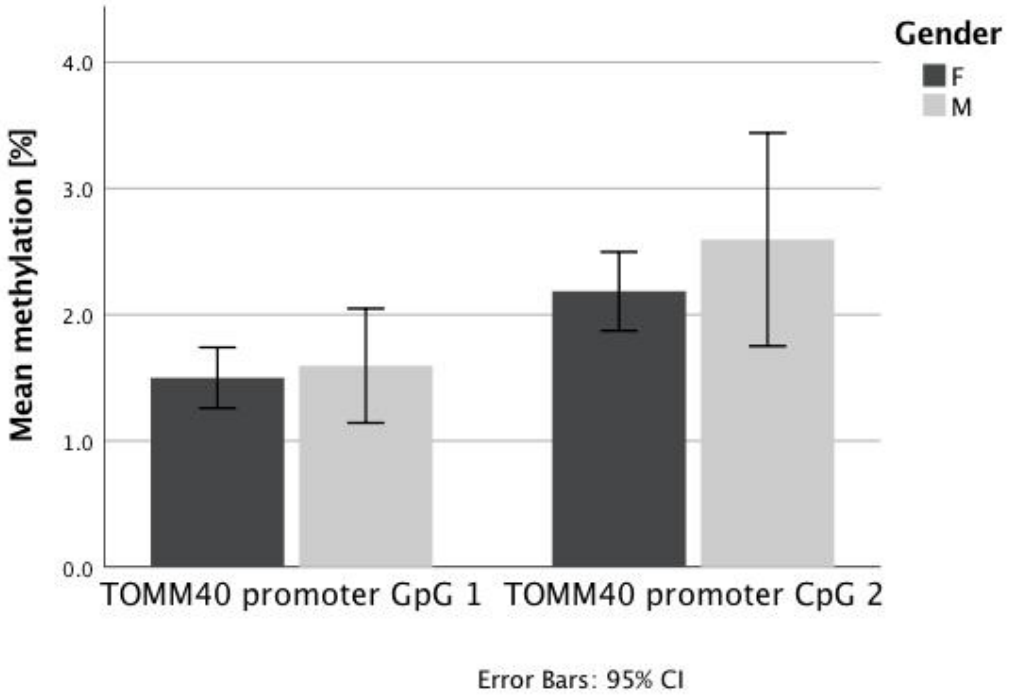

Figure 6 Mean methylation levels of the TOMM40 promoter region CpG sites stratified by gender.

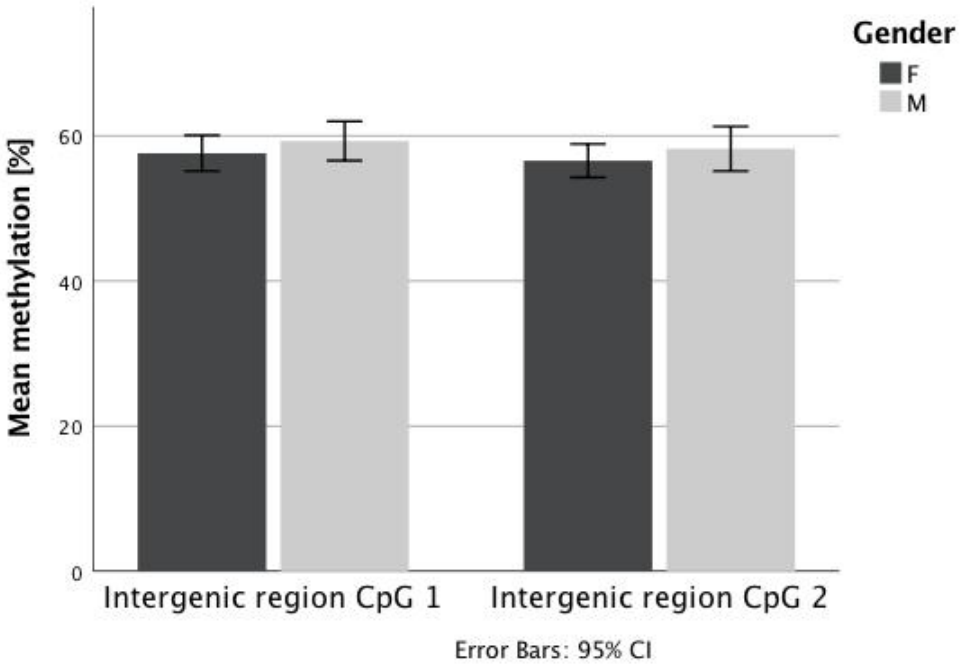

Figure 7 Mean methylation levels of the intergenic region CpG sites stratified by gender.

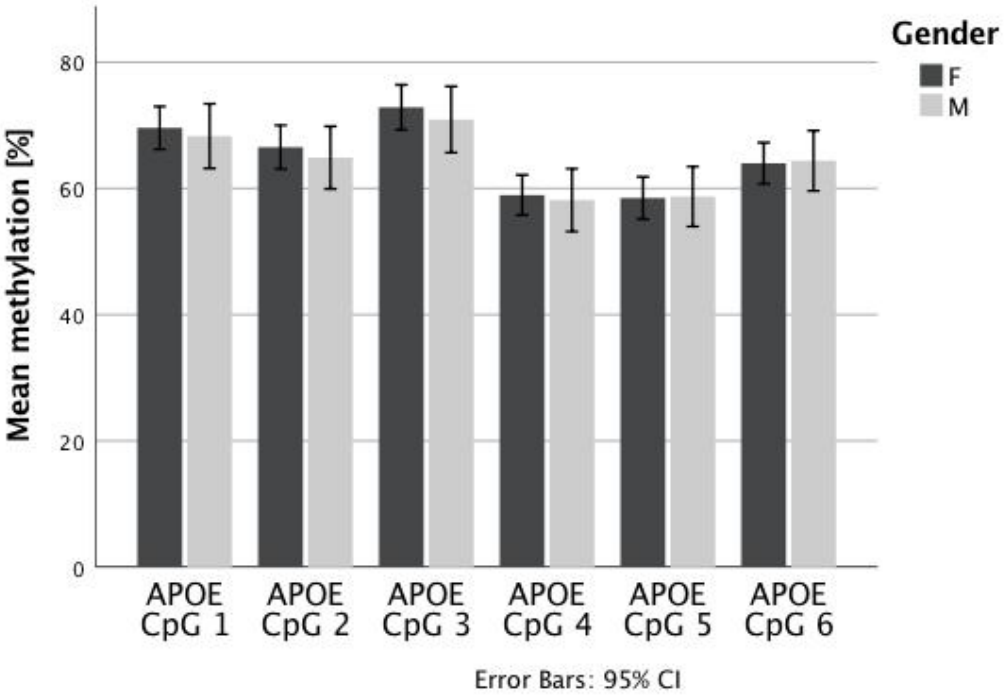

Figure 8 Mean methylation levels of the APOE exon 4 region CpG sites stratified by gender.

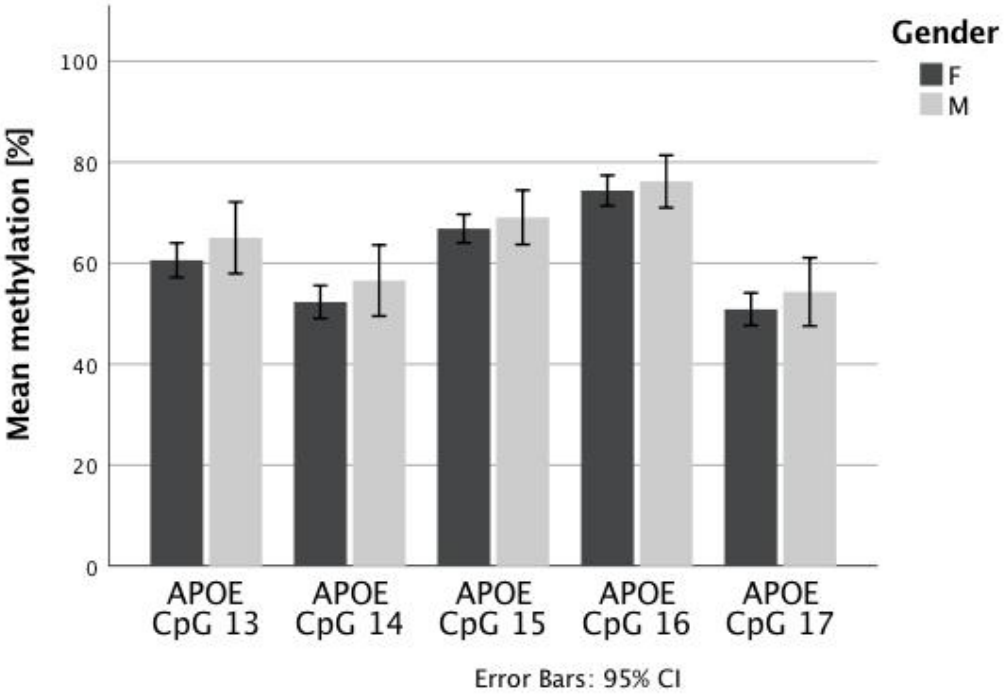

Figure 9 Mean methylation levels of the APOE exon 4 region CpG sites stratified by gender.

3.11 Methylation levels stratified by APOE4 allele presence and disease status (AD vs controls)

Next, it was tested whether the *APOE* genotype and the disease status might influence methylation of the locus. To do this, a two way ANOVA was performed with APOE genotypes, disease status set as grouping variables and DNA methylation at each of the CpG site across the *TOMM40-APOE* locus set as dependent variables and the results are shown in Table 11 . The test revealed that there were no significant differences in the brain APOE protein levels between the *APOE4* carriers vs *APOE4* non carriers in either AD or controls. None of the CpGs across *TOMM40-APOE* region methylation levels were significantly different between the genotype groups, or between AD and controls.

Table 11 Brain APOE protein levels and methylation levels stratified by APOE4 allele presence (carriers vs non-carriers) and disease status (AD vs controls). Data presented as mean (SD).

Variable	AD		Control		Effects (P-value)		
	APOE4 carrier	APOE4 non-carrier	APOE4 carrier	APOE4 non-carrier	Disease group	APOE Genotype	APOE Genotype x Disease group
TOMM40 CpG 1 methylation *	1.64 (1.1)	1.4 (0.5)	2.1 (1.5)	1.5 (0.8)	0.432	0.435	0.974
TOMM40 CpG 2 methylation	2.5 (2.3)	2.2 (0.7)	2.8 (2.2)	2.3 (0.7)	0.857	0.919	1.00
IR CpG 1 methylation	62.3 (4.9)	54.4 (6.5)	58.5 (8.0)	57.9 (8.7)	0.386	0.105	0.266
IR CpG 2 methylation	61.0 (4.9)	53.2 (5.5)	56.8 (8.1)	57.1 (8.5)	0.468	0.142	0.234
APOE CpG1 methylation	69.4 (6.0)	71.5 (8.4)	67.3 (18.1)	69.0 (9.3)	0.557	0.323	0.904
APOE CpG 2 methylation [%][a]	66.2 (6.8)	68.4 (8.9)	63.6 (17.6)	66.0 (9.3)	0.419	0.400	0.816
APOE CpG 3 methylation [%][a]	72.7 (6.5)	74.4 (8.6)	69.5 (18.4)	72.7 (9.9)	0.619	0.120	0.749
APOE CpG 4 methylation [%][a]	59.5 (6.7)	62.2 (8.5)	57.3 (16.3)	57.8 (8.4)	0.244	0.209	0.913
APOE CpG 5 methylation [%][a]	58.7 (6.6)	62.5 (8.9)	55.7 (16.3)	58.3 (8.4)	0.280	0.234	0.933
APOE CpG 6 methylation [%][a]	64.1 (7.3)	65.8 (9.8)	61.2 (16.5)	64.3 (8.4)	0.563	0.532	0.829
APOE CpG 13 methylation [%][b]	65.0 (13.1)	58.0 (11.3)	60.7 (20.8)	62.2 (9.9)	0.779	0.464	0.757
APOE CpG 14 methylation [%][b]	57.0 (12.7)	49.0 (10.5)	50.6 (18.7)	53.8 (9.7)	0.683	0.458	0.500
APOE CpG 15 methylation [%][b]	71.7 (10.1)	66.1 (8.3)	61.9 (19.0)	66.8 (7.2)	0.548	0.237	0.308
APOE CpG 16 methylation [%][b]	78.2 (11.3)	73.7 (6.8)	69.1 (19.7)	74.9 (7.9)	0.665	0.140	0.328
APOE CpG 17 methylation [%][b]	57.4 (11.9)	49.2 (10.1)	45.6 (17.7)	51.0 (9.9)	0.788	0.117	0.194

[a]data available in n=62
[b]data available in n=52
[c]data available in n=58
[d]data available in n=66

3.12 Correlation of the *TOMM40-APOE* locus methylation with the APOE protein levels

To investigate how the levels of methylation could influence the APOE protein expression, Pearson correlation test was performed (Table 12). This revealed that TOMM40 CpG site 2 methylation was negatively correlated with APOE protein level (ρ=-0.270, P=0.042). To account for confounding factors, the relationship between TOMM40 CpG2 methylation and APOE protein level was further investigated using linear regression model. APOE protein level was selected as the dependent variable and TOMM40 promoter region CpG2 methylation, age at death and APOE4 allele were set as independent variables. After adjustment for confounding factors, the association of TOMM40 CpG2 and the APOE protein level was no longer significant (B=-0.228, P=0.091).

Table 12 Pearson correlation of the TOMM 40 IR and APOE DNA methylation with APOE protein levels

Variable	ApoE protein level
TOMM40 CpG 1 methylation *	**-0.030**
TOMM40 CpG 2 methylation	**-0.270***
IR CpG 1 methylation	**0.183**
IR CpG 2 methylation	**0.193**
APOE CpG1 methylation	**-0.084**
APOE CpG 2 methylation [%][a]	**-0.074**
APOE CpG 3 methylation [%][a]	**0.066**
APOE CpG 4 methylation [%][a]	**-0.157**
APOE CpG 5 methylation [%][a]	**-0.156**
APOE CpG 6 methylation [%][a]	**-0.177**
APOE CpG 13 methylation [%][b]	**-0.175**
APOE CpG 14 methylation [%][b]	**-0.112**
APOE CpG 15 methylation [%][b]	**-0.115**
APOE CpG 16 methylation [%][b]	**0.055**
APOE CpG 17 methylation [%][b]	**0.004**

*correlation was significant at P<0.05

3.13 Correlation of the *TOMM40-APOE* locus methylation with AD pathology scores

To investigate if the methylation levels of the tested CpG sites associate with AD pathology scores (Thal, Braak and CERAD), Spearman rank correlation test was performed (Table 13). No significant associations were found between the methylation levels at any other investigated CpG sites and the APOE protein level. Thal, Braak and CERAD scores were not significantly associated with either of the CpG site methylation or APOE protein level.

Table 13 Spearman correlation between ApoE protein level or AD pathology and methylation levels.

Variable	Thal score	Braak score	CERAD score
TOMM40 CpG 1 methylation	**-0.104**	**-0.193**	**-0.127**
TOMM40 CpG 2 methylation	**-0.173**	**-0.219**	**-0.210**
IR CpG 1 methylation	**0.059**	**-0.108**	**-0.054**
IR CpG 2 methylation	**0.021**	**-0.129**	**-0.079**
APOE CpG1 methylation[a]	**0.024**	**0.070**	**-0.056**
APOE CpG 2 methylation [%][a]	**0.060**	**0.042**	**-0.037**
APOE CpG 3 methylation [%][a]	**-0.026**	**0.043**	**-0.043**
APOE CpG 4 methylation [%][a]	**0.064**	**0.059**	**-0.047**
APOE CpG 5 methylation [%][a]	**0.074**	**0.064**	**-0.028**
APOE CpG 6 methylation [%][a]	**0.057**	**0.019**	**-0.060**
APOE CpG 13 methylation [%][b]	**-0.079**	**-0.029**	**-0.109**
APOE CpG 14 methylation [%][b]	**-0.065**	**-0.028**	**-0.125**
APOE CpG 15 methylation [%][b]	**0.098**	**0.064**	**-0.007**
APOE CpG 16 methylation [%][b]	**-0.020**	**-0.016**	**-0.047**
APOE CpG 17 methylation [%][b]	**0.112**	**0.054**	**0.002**

[a]data available in n=62
[b]data available in n=52

3.14 Correlation of the *TOMM40* - *APOE* locus methylation with cognitive scores

Pearson correlation test was performed to investigate if methylation of the CpGs across *TOMM40-APOE* locus associate with cognitive scores and the results are shown in Table 14. This revealed that *APOE* CpG4 methylation level negatively correlated with longitudinal fluid intelligence (R=-0.278, P=0.046) and longitudinal vocabulary (R=-0.280, P=0.045), CpG 5 with cross sectional memory (R=-0.274, P=0.049, and longitudinal vocabulary (R=-0.288, P=0.039), whereas CpG 6 with longitudinal fluid intelligence (R=-0.275, P=0.047). TOMM40 CpGs 1 and 2 were significantly negatively associated with cross sectional fluid intelligence (R=-0.318, P=0.009), whereas only CpG2 with longitudinal fluid intelligence (R=-0.243, P=0.048).

Table 14 Pearson correlation between the cognitive measures and APOE protein levels and methylation levels. (The test was performed on both AD and control groups collectively).

Variable	Variable	CS fluid intelligence	CS vocabulary	CS speed	CS memory	L intelligence	L vocabulary	L speed
APOE CpG1 [a]	-0.126	-0.206	-0.037	-0.215	-0.210	-0.220	-0.130	-0.149
APOE CpG 2 [%][a]	-0.162	-0.225	-0.031	-0.214	-0.254	-0.231	-0.085	-0.120
APOE CpG 3 [%][a]	0.037	-0.196	-0.017	-0.216	-0.123	-0.219	-0.125	-0.158
APOE CpG 4 [%][a]	-0.211	-0.249	-0.090	-0.273	-0.278*	-0.280*	-0.092	-0.145
APOE CpG 5 [%][a]	-0.206	-0.261	-0.096	-0.274*	-0.258	-0.288*	-0.120	-0.145
APOE CpG 6 [%][a]	-0.209	-0.188	-0.044	-0.126	-0.275*	-0.185	-0.088	-0.054
APOE CpG 13 [%][b]	-0.124	0.068	0.068	-0.006	-0.041	0.065	0.129	0.025
APOE CpG 14 [%][b]	-0.099	0.061	0.062	0.002	-0.001	0.078	0.116	0.037
APOE CpG 15 [%][b]	-0.152	0.099	-0.079	-0.072	-0.063	0.053	-0.032	0.015
APOE CpG 16 [%][b]	-0.180	0.108	-0.082	-0.136	-0.092	0.057	-0.080	-0.031
APOE CpG 17 [%][b]	-0.088	0.121	-0.043	-0.049	0.007	0.122	-0.023	0.024
TOMM40 CpG 1 *	-0.318**	-0.028	0.046	-0.003	-0.186	0.039	0.085	-0.018
TOMM40 CpG 2	-0.318**	-0.048	-0.103	0.116	-0.243*	0.076	0.119	0.049
IR CpG 1	0.077	0.195	0.122	0.032	0.057	0.117	0.111	-0.071
IR CpG 2	0.089	0.162	0.100	0.069	0.065	0.077	0.064	-0.029

[a]data available in n=62
[b]data available in n=52
CS-cross sectional, L-longitudinal.

Linear regression analysis was performed to further investigate the association between longitudinal fluid intelligence and the methylation levels at the CpGs that significantly correlated. To account for other potential confounders, age at cognitive testing and *APOE4* allele presence were also included in the model as independent variables alongside the APOE protein level and *TOMM40* CpG2 methylation, *APOE* CpG4 and CpG6 methylation, while longitudinal fluid intelligence was set as dependent variable. After adjusting for confounding factors, only TOMM40 CpG2 methylation (B=-0.283, P=0.045) remained significantly associated with longitudinal fluid intelligence (Table 15). In case of the cross sectional fluid intelligence, linear regression model including both *TOMM40* CpG sites methylation levels could not be performed, as their significantly correlated with each other at R>0.7, which caused violation of assumptions for the linear regression model. When only CpG1 was included in the regression analysis, together with age at cognitive testing and APOE4 allele presence as confounding factors, the CpG 1 methylation level remained significantly correlated with cross sectional fluid intelligence (B=-0.305, P=0.014), however the overall model was not significant (P=0.052). Similarly in case of the Cross-sectional memory, remained significantly associated with APOE CpG5 methylation (B=-297, P=0.036) after adjusting for age at cognitive testing and APOE4 allele presence, however the overall proposed regression model was not significant.

In case of the longitudinal vocabulary, linear regression could not be performed as the dependent variable was not normally distributed.

Table 15 Multivariate linear regression analysis of longitudinal fluid intelligence with TOMM40 CpG2 methylation and APOE protein levels including potential confounders. Model summary: P=0.022, N=48, Adj R^2=0.188 R^2=0.291

Variable	Parameter estimate (Beta)	P-value
TOMM40 CpG2	-0.283	0.045*
APOE protein level	0.279	0.049*
Age at cognitive testing	-0.235	0.097
APOE CpG4	-0.351	0.416
APOE CpG6	0.316	0.474
ApoE4 allele	0.153	0.271

3.15 Immunohistochemistry staining

The immunohistochemistry staining revealed that APOE was present in the prefrontal cortex sample of both AD and control brains (green on Figures 10 and 11). However, the precise localisation of the protein APOE was not possible using this method, partially due to insufficient co-staining with neuronal cell markers. Moreover, the GFAP marker, used to identify astrocytes, indicated that the substantial amount of the tissue was degraded. Due to a substantial cell degradation in both AD and control samples, further analysis was not possible. For all these reasons, no differences in the IHC image between AD and control tissues could be identified in the present study.

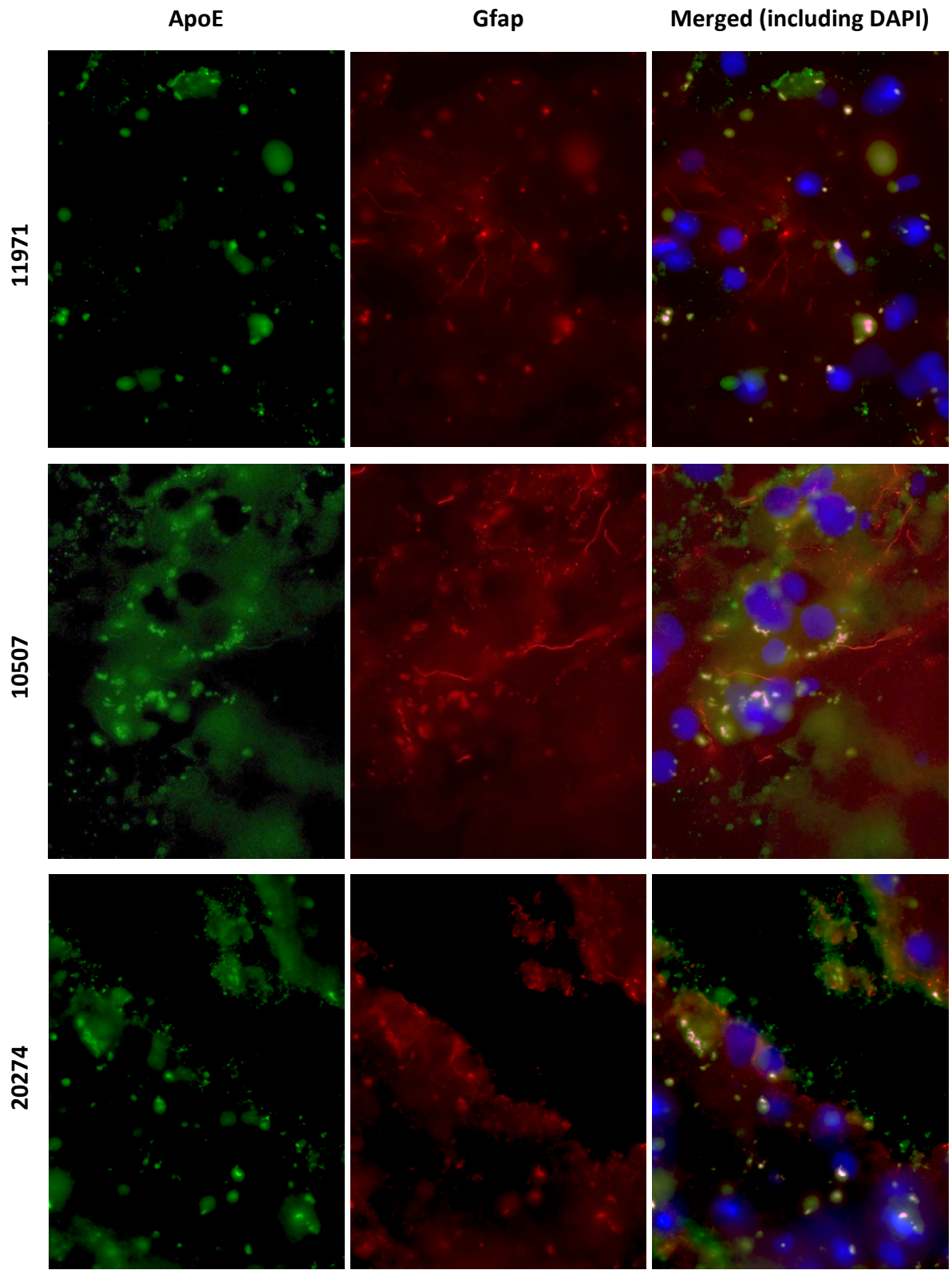

Figure 10 Immunohistochemistry staining of the APOE protein (green) protein, GFAP (red) and merged image (including DAPI nuclei stain) in the prefrontal cortex tissue sections in AD (x630 magnification)

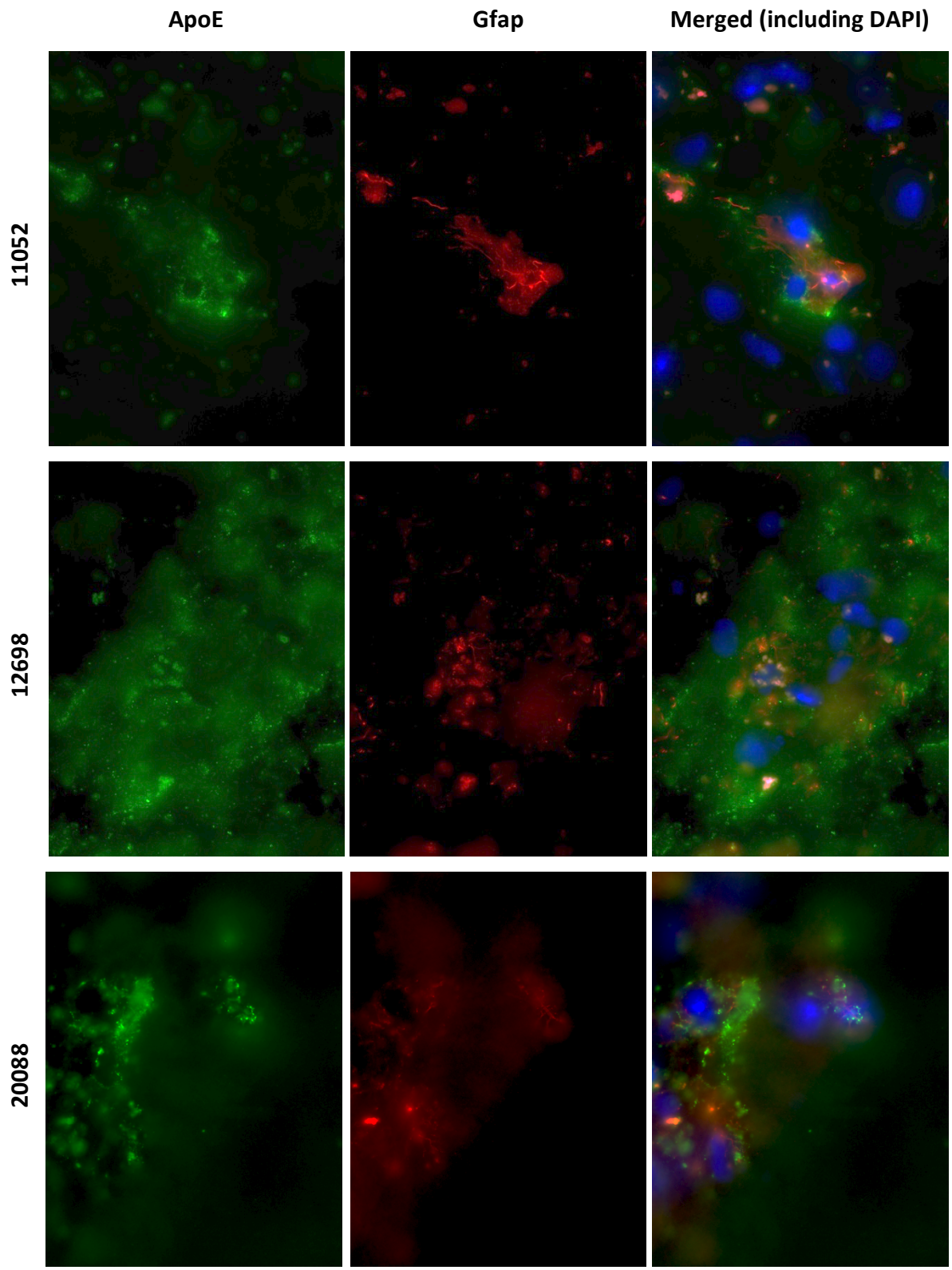

Figure 11 Immunohistochemistry staining of the APOE protein (green), GFAP (red) and merged image (including DAPI nuclei stain) in the prefrontal cortex brain tissue sections in controls (x630 magnification)

4. Discussion

The results of this project show that the number of *APOE4* carriers was higher among AD group compared to controls, however the total APOE protein level in the prefrontal cortex was not significantly different between the genotype groups or disease groups, indicating that the *APOE* genotypes may modulate AD risk independently of the total APOE protein expression. APOE protein level did however, positively correlate with longitudinal fluid intelligence, but not with vocabulary, speed or memory, suggesting that APOE protein may have a domain specific effect in the prefrontal cortex. Interestingly, longitudinal fluid intelligence was also negatively associated with Thal score, supporting the role of the previous mental abilities as a risk factor in AD. *TOMM40* CpG2 methylation levels were initially negatively correlated with APOE protein levels, however the significance was lost after adjustment for confounding factors. The remaining CpG sites tested in this study did not significantly correlate with the APOE protein expression. The *APOE4* genotype did not have an effect on the methylation levels at any of the CpG sites investigated. *TOMM40-APOE* locus methylation was not significantly different between AD and control, nor did it correlate with any of the AD pathology scores. *TOMM40* CpG2 methylation was significantly negatively correlated with longitudinal fluid intelligence, suggesting that the possible mechanism of domain-specific effect of the APOE protein levels on cognition may be related to altered methylation levels in the regulatory region.

4.1 Differences between AD and controls

The number of the *APOE4* carriers was significantly higher in the AD cases compared to controls. It aligns with previous findings, as it has been widely established that carrying at least *APOE4* allele significantly increases the risk of sporadic LOAD (Corder *et al.*, 1993; Blacker *et al.*, 1997). There are multiple proposed mechanisms by which *APOE4* allele could increase the risk of the AD. For instance, APOE 4 may enhance amyloid plaque formation, by forming stable complexes with Aβ (Strittmatter *et al.*, 1994; Cho *et al.*, 2001) or reduce Aβ clearance via dysregulation of astrocyte colocalisation, which reduces their ability

to clear Aβ (Koistinaho et al., 2004). The E4 allele may also contribute to AD pathogenesis independently of AB. It may for example, enhance tau phosphorylation (Cruchaga *et al.*, 2013) or cause neurotoxicity via mitochondrial or cytoskeleton disruption The partially degraded APOE4 particles may also accumulate in the neurofibrillary tangles (P. B. Jones *et al.*, 2011; Huang *et al.*, 2001).

However, the presence of the APOE4 allele is not sufficient nor is it necessary for the disease development and there must be other mechanisms involved in the AD pathogenesis. The other risk factors may include different genes that act in combination or separately from *APOE* genotype. There are also certain environmental determinants that have been identified as potential risk factors. This research hypothesised that these may affect gene methylation and therefore the protein expression (acting in line with or separately from the genotype).

4.2 APOE protein levels in AD compared to controls

In the present research, AD and the *APOE4* allele presence did not have a significant effect on the APOE protein levels, nor did the interaction between the two variables. Furthermore, APOE protein levels did not significantly correlate with any of the AD pathology scores (Braak, Thal and CERAD). However, only the total APOE levels were measured, not the levels of the specific APOE isoforms. This could mean that the overall APOE levels do not significantly affect the AD pathology development and the effect is isoform-specific. Alternatively, it could be the case that the overall APOE protein level in the brain does not influence AD whatsoever. However, it is unlikely, as previous research suggested that APOE has a neuroprotective role (Toledo *et al.*, 2014), aiding Schwann cells remyelination and axons recovery upon tissue injury (Huang and Mahley, 2014), indicating that decreased levels could lead to aggravated neurodegeneration and cognitive decline. There is also a number of other properties of the APOE2 and APOE3 that might be protective of AD. Multiple possible mechanisms of the APOE4 contribution to the AD pathology have been presented, and a number of studies implicated altered APOE protein levels directly in the brain or CSF as a contributor to the

pathology. Previous findings show that AD would typically be associated with decreased APOE protein levels in the frontal cortex and hippocampus (Beffert *et al.*, 1999; Bertrand *et al.*, 1995). Again, it could be also a case that this study lacks statistical power to detect an association where it in fact present. Sporadic AD is a heterogeneous disease and various factors contribute to their pathogenesis. In certain circumstances these factors might have a more pronounced effect, in others they might only confer a small change. Moreover, the protein levels were only measured in prefrontal cortex and there could be more pronounced differences in the different brain areas such as hippocampus, which is predominantly responsible for the episodic memory, the first cognitive feature to be affected by AD.

4.3 APOE protein expression across *APOE* genotypes

The APOE genotypes were in the Hardy - Weinberg equilibrium and the observed allele frequencies were not significantly different from expected for the British population according to the 1000 genome's project (The Genomes Project, 2015). Even though, the study sample was an adequate representation of the allele distribution in the population, due to relatively small sample size and relatively low minor allele frequencies for both genotypes, the data was stratified on the basis of the *APOE4* allele presence or absence alone, rather than according to the individual genotypes. If the homozygous genotypes would be compared, it could give an insight into how each allele affects gene methylation and expression individually; however, there were only two homozygous samples for the *APOE2* and *APOE4* genotypes, therefore the participants were grouped into *APOE4* carriers and *APOE4* non-carriers.

APOE protein levels were not significantly different between *APOE4* carriers and non *APOE4* carriers. This could mean that the *APOE* genotype does not affect protein expression in the brain prefrontal cortex. These findings differ from the previous publications, as other studies suggest that *APOE* genotype could affect APOE protein expression. *APOE4* allele has been previously associated with decreased APOE protein levels *in vitro* on the human astrocytoma cell culture (Riddell *et al.*, 2008), and *in vivo* in the mice brains as well as post mortem human

frontotemporal lobe brain samples (Bray *et al.*, 2004). However, the absolute levels of the APOE3 were similar between carriers of E3/3 and E3/4 genotype, suggesting that the overall decrease in the APOE protein levels could be due to the reduction in the E4 isoform. According to Riddell *et al.,* APOE4 has a shorter half-life and is degraded at a higher rate than APOE3, which might be the cause for overall decreased APOE protein levels in the *APOE4* carriers (Riddell *et al.*, 2008). The reason for the differences in findings could be the methodology differences between the studies, as the mouse and astrocytoma cell culture study only investigated the differences between the homozygotes for each allele, whereas in this study, both homozygous and heterozygous E4 allele carriers were compared against non-carriers. There could be also differences in the genotype effect between human samples and mouse or *in vitro* models. Alternatively, it could be the case that the present findings could be a false null due to the low sample size. Furthermore, an isoform-specific APOE protein quantification would allow to assess the effect of the individual genotypes more accurately.

4.4 Allele specific *APOE* methylation

It was hypothesised that the *APOE* exon 4 region methylation level would differ between the genotypes, especially because the rs7412 (C->T) removes a CpG site, and the rs429358 (T->C) creates an extra CpG site. This study, however, did not find any significant difference between the methylation levels of the *TOMM40* promoter region*, APOE* exon 4 CpG island or the intergenic region between *APOE* genotype groups. Again, a further stratification between the individual genotypes or best, investigation of differences between the homozygous samples could provide a better understanding on whether and how the *APOE* polymorphisms influence the gene methylation. There was one previous study, which suggested that the *APOE* exon 4 CpG island methylation was significantly higher in the frontal lobe among the carriers of E3/E4 genotypes compared to the E3/E3 carriers (Foraker *et al.*, 2015). However the effect was only seen in the controls but not in the AD and the sample size was relatively small (n=25). Other previous studies that investigated the *APOE* locus methylation did not observe any genotype effect on

the methylation levels (Karlsson *et al.*, 2018) or they did not investigate the difference in methylation between different genotypes (Shao *et al.*, 2018; Tulloch *et al.*, 2018). Therefore, further research, preferably in a larger cohort is necessary to fully understand how different *APOE* alleles may influence the methylation. Multiple environmental factors, such as diet, exercise, smoking, long term stress exposure, that were not available for this cohort may influence the gene methylation (Abdul *et al.*, 2017; Lee and Pausova, 2013). Accounting for these, for instance by stratifying the cohort according to theirs smoking status, could help to understand how the genotype may affect the methylation levels in combination with these confounders. The *APOE4* allele is thought to provide an advantage in the environments where the food availability is scarce, by increasing the cholesterol absorption from the intestine (Corbo and Scacchi, 1999). Therefore, in populations where the diet is high in calories and rich in saturated fat and refined sugar, carrying the E4 allele might be a disadvantage.

There are three possible mechanisms in which a SNP can affect gene methylation and expression: influence the methylation levels leading to altered gene expression, affect the gene expression which subsequently alters the methylation levels or it could affect both methylation and gene expression independently of each other (M. J. Jones et al., 2013). If a polymorphism alters or interferes with a transcription factor binding site, it is likely that it would affect both gene expression and methylation independently (Gutierrez-Arcelus *et al.*, 2013). As the transcription factors typically bind at the promoter region, it might be a case that there are no transcription factor binding sites in the exon 4 of the APOE gene, where the investigated SNPs are located and therefore, the *APOE* genotypes do not directly influence the levels of gene expression or methylation.

4.5 Brain APOE protein level and *TOMM40-APOE* region methylation association with AD pathology

Epigenetics could be an important aspect of the AD pathogenesis, especially considering that AD is very heterogeneous and it may exhibit very different clinical presentation even in patients with very similar genetic background (Yokoyama *et*

al., 2017). A good example of this could be a study on monozygotic twins, that showed a significant reduction of the methylation levels in the frontal cortex of the AD twin compared to the twin that did not develop the disease (Mastroeni *et al.*, 2009). Although, other studies have suggested that individuals with AD tend to have higher global methylation in the prefrontal cortex than controls (Coppieters et al., 2014; Rao *et al.*, 2012).

In the present study, methylation of the investigated *TOMM40-APOE* region in the prefrontal cortex did not significantly correlate with the APOE protein expression, nor did it correlate with any of the AD pathology scores. This area of research is quite novel and not many previous studies investigated the methylation of this specific locus in AD. A study that investigated the methylation of a number of AD-related genes in the temporal lobe, parietal lobe and the cerebellum did not find a significant difference in the *APOE* gene methylation between the AD and controls (Iwata *et al.*, 2014). Another study showed a significantly higher *APOE* promoter methylation in the prefrontal cortex samples of the AD patients compared to controls (Wang *et al.*, 2008). However, likewise in this study, there was no significant difference in the exon 4 CpG island methylation levels between AD and controls (Karlsson *et al.*, 2018). They did not specify which individual CpG sites in that region they investigated. Interestingly, the *APOE* exon 4 CpG island was hypermethylated, and the methylation levels across the regions were close to 100% in majority of cases. The *APOE* exon 4 CpG island was also hypermethylated in the present cohort, however the levels were much lower and there was more variation between each CpG sites ranging from 52-75% methylation. Although, there could be variation in methylation levels among different population, as they only investigated a small number of samples (n=34) and to account for these variations in the epigenetic studies, preferably a larger cohort would be required. It is worth mentioning that they also used a different method for the methylation levels quantification (MALDI-TOF), which could also be a possible cause for differences. There is, however, some evidence that the *APOE* exon 4 CpG island might be differently methylated in AD. For instance Foraker *et al.* (2015) found significantly reduced methylation in the hippocampus and frontal lobe but not the cerebellum of the AD brains, with the highest difference being in the frontal lobe. This finding

differs significantly from our findings however, *Foraker et al.* only analysed 25 samples and there could be covariates, such as cholesterol levels that were not accounted for, that might affect the result independently of the AD, that would influence the trend. Notably, they also used bisulphite pyrosequencing to measure the methylation levels and the mean methylation of the prefrontal cortex was 77%, which is closer to our findings, however significantly lower than in the Wang's study. These variations suggest that preferably a larger cohort would be required to investigate the effect of methylation on AD, especially if the results were to be applied to wider populations, and used as potential diagnostic/prognostic factors, as there may be different environmental determinants that affect methylation levels in the specific populations that would ideally need to be accounted for. One study investigated the *APOE* exon 4 CpG island methylation in AD and the Lewy Body Dementia (LBD) (Tulloch *et al.*, 2018) as *APOE4* has been also implicated as a risk factor in LBD (Lane *et al.*, 2009; Bras *et al.*, 2014; Keogh *et al.*, 2016). They found no significant differences in the methylation levels in the cerebellum, however there was a significant reduction of the methylation levels in the prefrontal cortex of the LBD brains. Interestingly, the methylation levels were even lower in the brains that exhibited both LBD and AD pathological changes, which is an intriguing finding, considering that both diseases share some clinical features and the rate of cognitive decline is more rapid if both AD and LBD occur concomitantly (Kraybill *et al.*, 2005; Chung *et al.*, 2015). In contrast to this study, neither Wang's, nor Tulloch's studies measure the APOE protein levels in the brains to compare if and how the methylation levels would affect the protein expression in the specific brain regions.

Two previous studies also investigated the extended *APOE* locus methylation in context of AD (Shao *et al.*, 2018; Karlsson *et al.*, 2018). According to Karlsson *et al.* (2018) findings, increased level of the *APOE* promoter methylation raises the odds of developing dementia and AD but not cardiovascular disease. Although, the methylation levels were not dependent on the *APOE* genotype. The study was conducted on a larger cohort of 447 Swedish twins and they adjusted the results for age, sex, education level, smoking and relatedness among twins and this study has a lot of strengths in terms of design. However they measured the levels of

methylation in the blood samples, which may differ from that in the brain, as it has previously found that the methylation levels of this region would vary across different tissue types (Shao *et al.*, 2018). Similarly, Shao *et al.* found a significant association of the *TOMM40-APOE* locus methylation and AD. Their findings indicate that *TOMM40-APOE* locus might be differently methylated in AD brains but not in the peripheral blood. Moreover, *APOE*, but not *TOMM40* methylation levels differed across different tissue types in both AD and controls. The proposed mechanism of action of the altered *TOMM40-APOE* methylation levels in AD was via the regulation of the APOE mRNA expression. They suggested that methylation of the *TOMM40* promoter region is correlated with both *TOMM40* and *APOE* gene expression and at certain CpG sites the correlations were of opposing nature: negative with the *TOMM40* gene and positive with the *APOE* gene. Furthermore, they found that another CpG site in the *TOMM40* promoter was associated with *APOE* mRNA expression, although the association was negative in controls and positive in AD. They did not, however measure the APOE protein levels in their study, which may be the cause for the differences in the findings, as this study measured APOE protein levels, but not mRNA levels. In the present study there was also a negative correlation of one of the *TOMM40* promoter region CpG sites with APOE protein expression, however we tested the association across the whole cohort rather than separately in AD and control groups and the significance was lost after adjusting for age at death and *APOE4* allele presence. All these findings indicate that *TOMM40-APOE* locus methylation might affect the AD pathology via altering APOE expression, however the specific mechanisms of these associations remain unknown. To be able to conduct epigenetic studies with a higher statistical power, ideally a larger cohort should be used, which would allow for appropriate stratification and perhaps this would help to detect the true associations.

4.6 Brain APOE protein levels, cross sectional and longitudinal cognitive scores and how do they relate to AD pathology scores

Our findings suggest that APOE protein levels in the prefrontal cortex positively correlate with longitudinal fluid intelligence ($R=0.265$, $P<0.05$) but not with the

cross sectional fluid intelligence. Although, the cognitive tests were performed earlier in their life and cross sectional intelligence could have changed by the time of death and sample collection. Therefore, the longitudinal cognition would be a better indicator of the overall cognitive performance for this type of correlation. Similarly to our findings, previous research suggested that APOE protein in the cerebrospinal (CSF) fluid may associate with longitudinal cognitive decline (Toledo *et al.*, 2014).They proposed a hypothesis that increased APOE2 and APOE3 expression in the CSF might be a protective response to a brain injury and accumulation of APOE protein in the brain may stimulate neuronal regeneration (Toledo *et al.*, 2014). The AOE protein levels did not, however, correlate with speed, memory or vocabulary. This could mean that APOE has a domain-specific effect in the particular parts of the brain, especially considering that dorsolateral prefrontal cortex is the main part of the brain involved in the fluid intelligence processes. It has been previously found that *APOE4* carriers that were also carriers of the Cathepsin D (CTSD) T genotype scored significantly lower in the fluid intelligence, but the *APOE* genotype did not have an effect when investigated separately (Payton et al., 2006). As APOE 4 isoform has been previously associated with decreased expression, it could suggest that *APOE* genotype could influence cognitive decline by altering APOE protein expression in the carriers of CTSD T genotype. Interestingly, methylation of the *TOMM40* CpG promoter region negatively correlated with longitudinal fluid intelligence. Initially, there was also a negative correlation between the *TOMM40* CpG methylation and the APOE protein levels, which could provide an insight into the mechanism of the association between the *TOMM40* methylation and longitudinal fluid intelligence (increased *TOMM40* promoter methylation$\rightarrow$ decreased APOE protein expression $\rightarrow$ decline of the fluid intelligence). However, the association of the *TOMM40* methylation with APOE protein levels was no longer significant after adjustment for confounding factors. Recent studies suggested that the *TOMM40* promoter region methylation may correlate with APOE protein expression in the hippocampus and cerebellum (Karlsson *et al.*, 2018; Shao *et al.*, 2018) . Nevertheless, these were only exploratory studies and the associations differed across the CpG sites. Moreover in one of the studies the nature of the correlation was opposite in the AD and controls (Shao *et*

al., 2018), therefore it would be difficult to establish what would be the overall effect of the methylation on the APOE protein expression in both groups collectively.

Although, the present study did not find any significant association of the *APOE4* genotype APOE protein levels in the prefrontal cortex and the levels did not significantly differ between AD and control brains, there are numerous studies that showed decreased levels of APOE protein in the AD brains and CSF and with a significant effect of the *APOE4* allele (Lehtimaki *et al.*, 1995; Merched *et al.*, 1997; Bekris *et al.*, 2010). As this study shows that the increased APOE protein levels did associate with better longitudinal fluid intelligence collectively in AD and controls, it may suggest that APOE could influence cognition independently of the AD development. However, longitudinal fluid intelligence also negatively correlated with Thal score. This is an interesting finding, as previous mental capabilities have been implied as a risk factor in AD (Mayeux, 2003; Mortimer *et al.*, 2003). All these findings suggest that epigenetic *TOMM40- APOE* regulation may influence cognitive abilities via regulation of APOE expression or via independent mechanisms and thus translate into AD pathology development in later life. Therefore, it is imperative to understand the mechanisms that regulate the APOE protein expression and may be potential therapeutic targets for the AD modulation. Due to loss of function and/or gain of toxic function effect of the *APOE4* allele, understanding how different alleles are transcriptionally regulated might be important in the early diagnosis and disease progression prediction.

4.7 Limitations and future recommendations

4.7.1 Limitations

This study has several strengths, including quantification of APOE protein levels in the brain as well as longitudinal cognitive scores that allow an insight into the AD risk factors and domain specific effect of *APOE* genotypes and the protein levels. Although, there are also several limitations to this study. For instance, a technical limitation is the method used for the methylation analysis, as bisulphite pyrosequencing performs best with very short sequences. In the runs involving a

longer sequence, the signal decreased or was lost towards the end of the sequence. To be able to determine methylation levels of the whole *APOE* gene regulatory region would require designing multiple assays that would be very costly and time consuming, which would significantly exceed the budget and time-scale of this project. Also, designing PCR and sequencing primers in the CpG-dense gene regions, as in case of the *APOE* exon 4 CpG island, was challenging, due to the possibility of unspecific binding in these areas or issues with primer compatibility. For this reason, the assay required a number optimization steps and more than one set of primers needed to be tested until the optimal results were achieved. If this method was to be further optimised for the purpose of analysis of *APOE* exon 4 region, to achieve sufficient yield in the PCR product and thus possibly higher signal in the pyrosequencing assay, it would be recommended to elute half of the volume of the of the converted bisulphite DNA than it was used in this study.

Another limitation occurred with the immunohistochemistry staining of the tissue sections. Examining the freshly cut sections under microscope, revealed that in certain samples the tissue was partially degraded and vacuolized. Possible cause of the damage could be sample handling at the brain centre, such as extended time between tissue collection and freezing.

With multifactorial diseases such as dementia and AD, individual SNPs or methylation of small gene region may confer only a modest influence on the pathology and the diseases risk, therefore, to avoid type II error larger sample size would be allow to detect whether there are any true associations. Due to a low sample size this study may lack power to detect any significant associations.

As a result of a significant post mortem delay, the levels of mRNA were very low and of poor quality, therefore, this study did not measure mRNA levels which could allow to give an insight into how *APOE* methylation directly affects the level of mRNA transcripts and which CpG sites may have a significant effect.

4.7.2 Future recommendations

Performing an isoform-specific high-sensitivity ELISA, would help to gain a better understanding of how different *APOE* alleles influence AD pathology. Going forward, an investigation of the methylation levels across the whole *TOMM40*-

APOE region, especially in the promoter regions could help to identify possible single CpG sites or islands that may have an effect on the protein expression and thus AD pathology. Furthermore, comparing the levels of the *APOE* mRNA and the transcription factors across the genotypes and AD pathology stages as well as correlating that to the methylation levels could provide more detailed insight on the allele-specific gene regulation in AD. As the mRNA production stops at the time of death, measuring the mRNA levels in blood and CSF could provide a better understanding of how different *APOE* alleles affect the gene expression and how *APOE* gene is methylated across different tissue types. To expand on the current research, the future studies could also measure levels of cholesterol in the brain. Comparing the cholesterol levels between different alleles and AD pathology stages, could provide an insight into the mechanisms by which APOE alleles may modulate AD development.

In regards to immunohistochemistry staining, co-staining with neuron specific antibodies (NEFM), and oligodendrocytes antibodies (CNP) would allow localisation of the APOE protein in the brain tissue. Furthermore, staining with isoform-specific APOE antibodies and comparing in the heterozygous samples could provide an insight into the localisation and ratio of the specific isoforms.

5. Conclusion

Sporadic AD is a complex disorder, displaying heterogeneous pathogenesis and numerous genetic variants together with environmental factors may interact to influence the diseases risk.

TOMM40-APOE locus methylation might influence the risk of AD by modulating APOE protein expression directly in the brain. Although, this study did not find any association of *TOMM40-APOE* region methylation, APOE protein expression and the AD pathology, there are some previous studies that suggest that this region is differently methylated in AD disease and the effect might be linked to *APOE* genotype and lead to altered *APOE* expression.

We found that APOE protein levels in the prefrontal cortex positively correlate with longitudinal fluid intelligence, which also correlates with Thal score. These findings indicate that APOE protein may have domain - specific effect on longitudinal cognition and therefore influence the AD risk possibly by affecting the deposition of amyloid plaques.

Further studies, preferably including a larger sample size are necessary to fully establish how methylation of *TOMM40-APOE* locus regulates APOE protein expression in the brain and how this influences the risk of AD.

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